Cátia Filipa Patrício Rodrigues

Licenciada em Ciências Biomédicas

Dissecting the role of the Ral/Exocyst pathway in postsynaptic growth and activity-dependent plasticity

Dissertação para obtenção do Grau de Mestre em

Genética Molecular e Biomedicina

Orientadora: Doutora Rita Teodoro, Investigadora Principal, CEDOC/FCM

Júri:

Presidente: Prof. Doutora Paula Maria Theriaga Mendes Bernardes Gonçalves

Arguente: Prof. Doutora Rita Oliveira Teodoro

Vogal: Prof. Doutora Cláudia Guimas de Almeida Gomes

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Novembro 2016

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Abstract

Neurons are the most morphologically diverse cell type whose morphology determines many functional aspects of a neuronal network. The primary shape of neurons is established during axon and dendrite outgrowth and synapse formation, but can subsequently be modified by synaptic activity. Postsynaptic compartments, such as dendritic spines or the postsynaptic membrane (called the Subsynaptic Reticulum or SSR) of the *Drosophila* Neuromuscular Junction (NMJ) are highly dynamic elements that are subject to this type of plasticity. The principal goal of this work is to define cellular and molecular mechanisms of synaptic growth and plasticity. We focus on a novel pathway that regulates neuronal morphology in response to activity through the engagement of Ral and the Exocyst complex in the regulation of membrane growth at the synapse, in response to neuronal activity. Since we know that Rab GTPases play a role in polarized vesicle delivery, we hypothesized that a subset of them will be required to mediate Ral/Exocyst-dependent structural plasticity. Using the *Drosophila* NMJ as a model synapse, we tested all Rab GTPases - by screening a collection of Rab GTPases RNAi and YFP-tagged Rab GTPases - for their capacity to mimic Ral- and exocyst-dependent effects on postsynaptic growth. We identified three candidate Rab GTPases that might mediate postsynaptic growth in a Ral/Exocyst-dependent manner. Our main interest is to dissect the genetic cascade that converts synaptic activity into postsynaptic membrane growth in a Ral/Exocyst-dependent manner, and how Rab GTPases and its regulators/effectors interact and regulate this mechanism. We believe that a deep understanding of the basic mechanisms and genes that regulate neuronal growth and plasticity will serve to uncover general principles that link normal development and function to dysfunction.

Keywords: Rab GTPases; Drosophila; Postsynapse; Ral/Exocyst; Neuronal Growth

Resumo

Os neurónios são o tipo celular com maior diversidade morfológica, e cuja forma determina muitos dos aspetos funcionais na rede neuronal. A forma primária dos neurónios é estabelecida durante a crescimento axonal e dendrítico e durante a formação de sinapses, podendo ser alterado pela atividade sináptica. Os compartimentos pós-sinápticos, como por exemplo, as dendrites ou a membrana pós-sináptica da junção neuromuscular (NMJ) da *Drosophila*, chamado retículo sub-sináptico (SSR), têm elevada plasticidade. O objetivo principal deste trabalho é tentar definir mecanismos celulares e moleculares de crescimento e plasticidade sináptica. Para tal, focámos a nossa atenção num mecanismo recentemente descoberto capaz de regular a morfologia neuronal em resposta à atividade através da via de sinalização do Ral/Exocisto. Esta via tem a capacidade de regular o crescimento membranar nas sinapses em resposta à atividade neuronal. As Rab GTPases são proteínas que desempenham funções importantes nos mecanismos de transporte membranar e polarizado nas células. Como tal, hipotetisámos que parte das Rab GTPases pudessem estar envolvidas neste mecanismo de crescimento pós-sináptico mediado pelo complexo Ral/Exocisto. Para responder a esta questão usámos a NMJ da *Drosophila* como modelo de atividade sináptica, onde testámos todas as Rab GTPases usando uma coleção de Rabs marcadas endogenamente com YFP e uma coleção com que expressa RNAi para testar os efeitos do *knockdown*.

Identificámos 3 Rab GTPases capazes de interferir no crescimento pós-sináptico via Ral/Exocisto. O nosso interesse principal é perceber os mecanismos genéticos envolvidos na conversão de atividade sináptica em crescimento do SSR. Estamos convictos de que um conhecimento profundo dos mecanismos básicos e dos genes que regulam crescimento neuronal e plasticidade servirá para descobrir os princípios que relacionam o desenvolvimento e função dos circuitos neuronais, com a disfunção que está presente em várias doenças do foro neuronal.

Palavras-Chave: Rab GTPases; Drosophila; Pos-sinapse; Ral/Exocisto; Crescimento Neuronal

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Abbreviations

µm	Micrometer
AU	Arbitrary units
CA	Constitutively active
Dlg	Discs large
dsRNA	Double-stranded RNA
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GEF	Guanine exchange factor
GFP	Green Fluorescence Protein
HRP	Horseradish Peroxidase: labels neurons
YFP	Yellow Fluorescent Protein
LTD	Long-term depression
LTP	Long-term potentiation
NMJ	Neuromuscular Junction
NGS	Normal Goat Serum
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Triton-X
PFA	Parafolmaldehyde
PHEM	PIPES-HEPES-EGTA-Magnesium
PIP	phosphatidylinositol (4,5)-biphosphate
Ral-GDS	Ral-GDP dissociation stimulator
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT	Room Temperature
SNARE	Soluble NSF-attachment protein
SSR	Subsynaptic Reticulum
TEM	Transmission Electron Microscopy
TRPL	transient receptor potential channel
UAS	Upstream Activating Sequences
VRDC	Vienna Drosophila RNAi Center

Chapter 1. Introduction

The development and maintenance of neural circuits is essential for the formation and function of the nervous system. Neurons are the most morphologically diverse cell type, and their morphology determines many aspects of function. In response to changes in synaptic activity, neurons can alter both pre- and postsynaptic elements of the synapse. Defects in synaptic morphology and activity-dependent plasticity are a hallmark of several neurodevelopmental and neurodegenerative disorders. It is therefore critical to know the basic mechanisms by which neurons acquire their shape and change it in response to activity. In the *Drosophila melanogaster* (from now on called *Drosophila*) neuromuscular junction (NMJ) the postsynaptic membrane - called the subsynaptic reticulum (SSR), is characterized by a set of folded membranes that are regulated by the Ral/Exocyst pathway in an activity-dependent manner. The nature of the vesicles that contribute for SSR growth or the genetic cascade that regulates their trafficking and fusion with the postsynaptic membrane are unknown. Because Rab GTPases coordinate most of the endocytic and exocytic membrane pathways, we asked if and which Rab GTPases contribute to this process. Understanding the pathways that regulate the growth of SSR can help answering some questions regarding synaptic growth and how structure is modulated by activity.

1.1. Regulation of Neuronal Growth

During development neurons extend their axon and dendrites to the target tissue in order to make the right synaptic connections with their partners. Being a central aspect of neurobiology, there are a number of studies focused on synapse formation, synaptic physiology, and circuit organization and function (Shen & Cowan 2010). Synapses are specialized functional and morphological cell structures that allow neurons to communicate with the receptor cells. Synapses are characterized by having a pre- and a post-synaptic side: in excitatory synapses, presynaptic terminals of neurons contain neurotransmitter-filled vesicles and the machinery necessary for its release, the postsynaptic side can either be another neuron or a specialized cell containing receptors to the neurotransmitters released, such as the muscle in the case of NMJs. Excitatory synapses are plastic, which means that terminal connections are able to modify their structure in response to neuronal activity (Menon et al. 2013). Synaptic plasticity involves structural and functional changes that are thought to be the foundation of learning and memory formation (Shen & Cowan 2010). For many years, the neuromuscular junctions of *Drosophila* have been used as a powerful model synapse because *Drosophila* NMJs are glutamatergic, with a stereotyped morphology that is genetically regulated but that can be modified by activity. In *Drosophila* larvae, the muscle and nerves grow from 1st to 3rd instar, increasing the muscle area ~100 times and the NMJ and the synaptic boutons over 10 times (Figure1.1) (Hill et al. 2015). The postsynapse of *Drosophila* larvae is a membranar structure that grows in an activity-dependent manner and understanding how the membrane traffic and activity are genetically connected might give us insights into the regulation of the molecular mechanisms that determine neuronal structure.

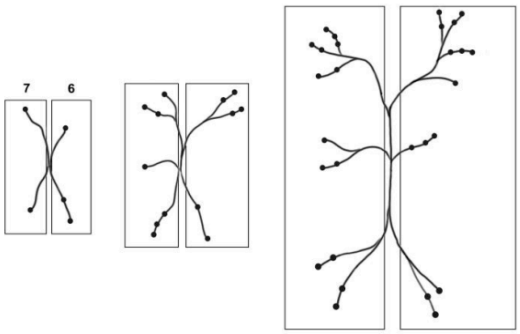


Figure 1.1. Development of Neuromuscular Junction (NMJ) in muscle 6 and 7 in *Drosophila* larvae from 1st instar to 3rd instar. With the elongation of the muscle NMJ increases in size and more synaptic boutons are added. Adapted from (Menon et al. 2013).

1.2. What Regulates Neuronal Morphology?

Neurons have a wide variety of shapes and sizes. To achieve and maintain neuronal morphology, and a large surface area, continuous membrane addition is required. The source of new membrane can come remotely from the cell body of the neuron, where most of the synthetic pathway machinery is located, or from local sources such as endosomes. Neurons are highly polarized cells that have distinct membrane domains, and long distances between sites of synthesis and of neurite growth. Thus, the process of neuronal membrane growth faces some challenges that no other cell type encounters (Pfenninger 2009).

Neuronal growth and their continuous expansion requires membrane trafficking and exocytosis (Wickner & Schekman 2008): defects in these mechanisms can lead to defects in neuronal structure and function, which can result in nervous system disorders (Pfenninger 2009). Regulation of membrane traffic events has also been associated with the capacity of neurons to strength synapses. The long lasting increase or decrease of synaptic strength, respectively known as long-term potentiation (LTP) and long-term depression (LTD), are intrinsically associated with cellular and molecular processes capable of altering the plasticity of synapses. During LTP expression, the rapid insertion of membrane and membrane proteins has to occur to support the enlargement of spines (Kennedy et al. 2010). Dysregulation of membrane traffic can block LTP due to problems with endosomal trafficking or exocytosis, and remodelling of the actin cytoskeleton. Besides being regulated by synaptic activity, all these processes are regulated, by small GTPases like Rab GTPases, in the case of membrane traffic, and by Rho GTPases in what concerns the remodelling of the actin cytoskeleton (Colgan & Yasuda 2014). The morphology of the *Drosophila* NMJ is also regulated by synaptic activity and by small GTPases. In *Drosophila* larvae the synapses of the NMJ have a presynaptic side with pools of vesicles containing neurotransmitters, called active zones, and opposite to that have a postsynaptic side formed by numerous convoluted folds and invaginations of membrane, called subsynaptic reticulum (SSR) (Harris & Littleton 2015). How the SSR size and shape is regulated is still poorly understood.

1.3. Regulation of membrane trafficking by small GTPases

Small GTPases are GTP-binding proteins that are present from yeast to human, constituting a superfamily with more than 100 members among 5 families, the Ras, Rho, Rab, Sar1/Arf and Ran. These families regulate a wide variety of molecular processes acting like biological switches and "biotimers", which associated with their regulators and effectors act as control elements in signalling pathways (Takai et al. 2001; Colicelli 2010). Small GTPases are monomeric G proteins that function as nucleotide-dependent switches that have affinity for both GDP and GTP, assuming different conformations in each case. These proteins can be in an active conformation (GTP-bound) or inactive conformation (GDP-bound) that are regulated by guanine exchange factors (GEF), which activates them, and by GTPase activating proteins (GAP) that promote the hydrolysis of GTP, inactivating the small GTPase (Novick 2016; Takai et al. 2001). These two interconvertible forms allow cycles of activation and inactivation that function as molecular switches to transduce upstream signals to downstream effector(s) (Figure 1.2).

A fraction of Small GTPases can be associated with membranes via one or two prenyl groups requiring a GDP dissociation inhibitor (GDI) to extract the inactive GTPase from membranes, masking the prenyl groups, and/or redeliver them to the target membrane (Pfeffer 2013; Mizuno-Yamasaki et al. 2012).

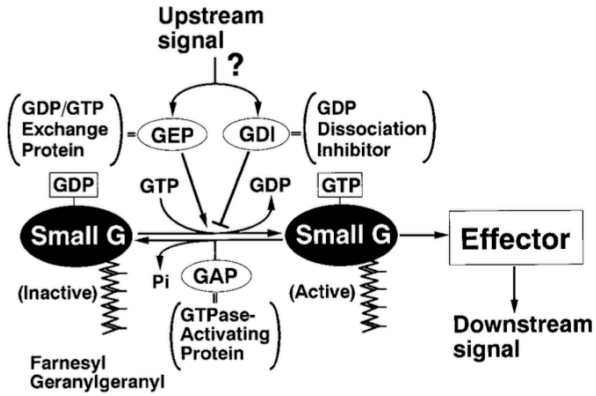


Figure 1.2. Cycle of regulation of Small GTPases. Small GTPases cycle between an active and an inactive form in the presence of upstream signal. An upstream signal stimulates the dissociation of GDP from the GDP-bound allowing GTP to bind. Alterations in the conformation of the Small GTPase can transduce the signal to effectors. Adapted from (Takai et al. 2001).

Organelles of the exocytic and endocytic pathways are linked by a rapid and bidirectional membrane trafficking flux mediated by vesicular transport. One of the challenges in the field is the understanding of how the identity of each organelle is maintained and how the selection of cargo is controlled, from the donor compartment to the receiver, prior to the fusion. Some Small GTPases can regulate both spatially and temporally the identity of compartments allowing the specific transport of cargo (Mizuno-Yamasaki et al. 2012). Sometimes, the delivery of membrane vesicles depends on a cascade of small GTPases that interacts with various effectors that can help and promote the motility of vesicles, like what it is described in figure 1.3. Canonically, Small GTPases require a GEF for activation. In some cases, the activation of one GTPase, like Rab GTPases, leads to the activation of

other GEF that can activate another Rab GTPase. This mechanism can activate a cascade of Rabs that are effectors for many processes in cells (Mizuno-Yamasaki et al. 2012).

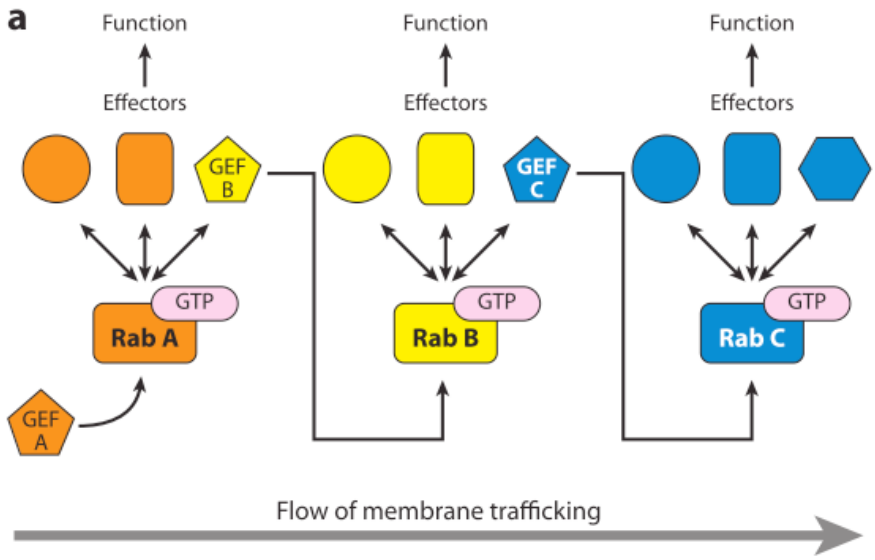


Figure 1.3. Membrane flux involves Rab GTPases activation. When a GEF activates one Rab GTPase, it makes that Rab suitable for activating many effectors and processes in cells. In the image a GEF cascade is described that by sequential activation of Rab proteins and GEFs promotes the traffic of membranar vesicles. Adapted from (Mizuno-Yamasaki et al. 2012).

Rab GTPases are a subset of small GTPases that give vesicles/organelles a specific identity that, by a mechanism of Rab GTPase conversion, allows vesicles to mature and progress through the endo or exocystic pathways (Pfeffer 2013).

1.3.1. Rab proteins as membrane traffic regulators

Rab proteins are one of the largest families of proteins within the small GTPases superfamily. Rab proteins are associated with most of vesicular trafficking pathways and are considered the major regulators of membrane vesicle traffic in cells, allowing specific delivery of cargo and membrane to the correct place (Takai et al. 2001; Pereira-Leal & Seabra 2001). Rab GTPases were first described by Touchot and his collaborators in 1987 when they isolated four family members from a rat brain cDNA library and consequently named them “rab” (Touchot et al. 1987). Rab GTPases have been found in all eukaryotes from *Saccharomyces cerevisiae*, through mammals and are normally found in cellular membranes, even though a small fraction can be cytosolic. In mammals there are about 70 Rab proteins (Zerial & McBride 2001; Colicelli 2010), 28 in *Drosophila* (Zhang et al. 2007), about 29 in *C. elegans* (Pereira-Leal & Seabra 2001) and 11 in *Saccharomyces cerevisiae* (Lazar et al. 1997). Their large number and wide distribution in several species highlights their importance in eukaryotic cell function (D’Adamo et al. 2014).

Rab proteins have been implicated in distinct pathways of membrane traffic and are necessary for vesicle budding, motility, tethering and fusion (Figure 1.4). All the flux of membrane traffic has to be regulated by a cascade of Rab proteins that controls where and when vesicles are trafficked, docked

and fused. For these events to occur, membrane vesicles have to acquire a specify Rab identity (Stenmark 2009). Today, it is well established that some Rabs are specifically associated with vesicles from endocytic and recycling pathways in cells, while others are associated with exocytic routes. For example, for the maturation of endosomes from early to late endosome, it is known that it is necessary to have a succession of events for maturation to occur. Rab5, is associated and a marker of early endosomes, and by a Rab-conversion mechanism the vesicles acquire the late endosomal marker Rab7, which allows them to mature into late endosomes (Stenmark 2009). Another example is the trafficking of vesicles from the endoplasmic reticulum to the Golgi apparatus that always seems to have Rab1 and Rab2 identity (Bhuin & Roy 2014). Rab1 is also a marker for vesicles exiting the Golgi (Mizuno-Yamasaki et al. 2012). The incapacity of vesicles to acquire a specify Rab identity leads to problems with vesicle traffic and human disorders like X-linked centronuclear myopathy, which is a severe congenital myopathy caused by intracellular β1-integrin accumulations in endosomes (Ketel et al. 2016).

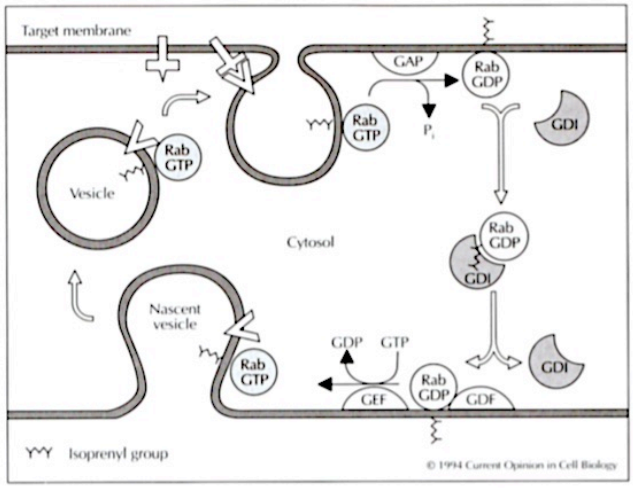


Figure 1.4. Vesicle formation and specific traffic mediated by Rab GTPases. Following the arrows and going clockwise in the figure, RabGTPases in the GDP form are recruited by GDI that guides them to a GEF to be converted in the GTP form. In this GTP form, Rabs are able to bind to vesicles giving them specific identity and allowing the specific membrane transport (Pfeffer 1994).

Besides vesicles, Rabs can interact with many different effectors like microtubule-motors, such as Rabkinesin-6 that interacts with Rab6 thereby promoting the delivery of vesicles from Golgi to endoplasmic reticulum (Echard et al. 1998). Rabs also interact with actin-dependent motors and motor-adaptors, like myosin-Va and melanophilin that bind Rab27a in melanosomes, mediating their transport to the periphery (Hume et al. 2007; Bahadoran et al. 2001). Another example is the interaction between regulatory complexes, such RIMα/Munc13/α-liprins and Rab3, that together regulate synaptic vesicle exocytosis (Schoch et al. 2002). Many structural analysis of Rab proteins show that they assume similar structures among all Rabs, but have different binding sites for distinct effectors allowing the selective recognition of diverse effector proteins (Pfeffer & Aivazian 2004).
In humans, mutations in Rab proteins can lead to different disorders (Bhuin & Roy 2014) like Charcot-Marie-Tooth type 2B characterized by distal muscle weakness, foot ulcers and infections due to

mutations in the late endosomal protein Rab7 (Verhoeven et al. 2003). Another example is Carpenter's Syndrome which is an autosomal recessive disorder characterized by craniosynostosis, obesity and cardiac defects that are caused by mutations in Rab23 which acts as a negative regulator of hedgehog signalling in cranial-suture development (Jenkins et al. 2007). These are just a few examples of how membrane and protein traffic regulation by RabGTPases can lead to human diseases - many more have been identified. It is therefore critical to understand how RabGTPases are regulated, which effectors they bind, and how they coordinate intracellular trafficking. Coordination of trafficking is a complex process that involves several steps and players. A protein complex that interacts with several distinct small GTPases, including several Rab GTPases, is the exocyst complex. By being able to receive regulatory information from different pathways, the exocyst can serve as a hub to precisely regulate where and when vesicles will fuse (Takai et al. 2001; Mizuno-Yamasaki et al. 2012).

1.3.2. The exocyst complex and its role in membrane trafficking

The exocyst is an octameric protein complex (Wu & Guo 2015) that was first described in the budding yeast *Saccharomyces cerevisiae,* in a genetic screen for temperature-sensitive secretory mutants (Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15) (Novick et al. 1980). Later, in a screen for exocytosis, two additional exocyst members were identified (Exo70 and Exo84) (TerBush et al. 1996). The crystal structure and bioinformatics prediction of several exocyst subunits showed that the subunits are mostly composed by α-helical bundles (Yu & Hughson 2010). Transmission Electron Microscopy (TEM) using "Quick-freeze" methodology, showed that the exocyst has a "T" or "Y" conformation that suggest that when the complex assembles, the subunits snap together to facilitate tethering to the membrane (Munson & Novick 2006; Hsu et al. 1998). Each subunit of exocyst has specific functions, and can interact with other effectors. It has been a matter of debate in the field whether the exocyst complex always functions as an octameric complex or whether sub-complexes may form. Based on different mutant phenotypes and on distinct localization of the proteins, it is likely that the exocyst can act in different populations, but the identity of these is still to be resolved (Heider & Munson 2012).

The exocyst is implicated in the tethering of secretory vesicles to the plasma membrane in sites of active exocytosis and membrane expansion, where it mediates tethering, prior to SNARE(Soluble NSF-attachment protein) -mediated fusion (Wu & Guo 2015). The exocyst complex is able to assemble and tether vesicles at sites of fusion through interactions with small GTPases that are spatially activated (Novick & Guo 2002; Chen et al. 2011). There are studies that describe the interactions of the exocyst with Rab proteins (Guo et al. 1999), with Rho proteins (Guo et al. 2001) and with Ral proteins (Moskalenko et al. 2002; Sugihara et al. 2002). In Figure 1.5 is showed a schematic representation of the exocyst complex attached to a vesicle.

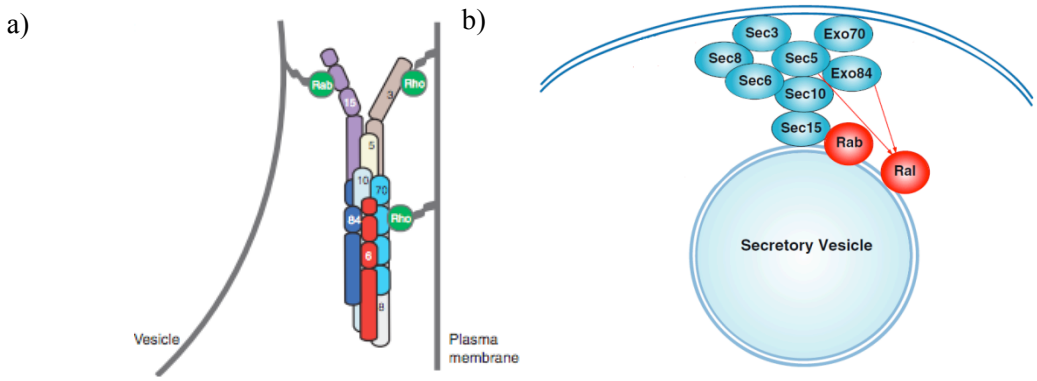


Figure 1.5. Scheme of exocyst complex. a) Schematic representation of the structure of Exocyst, in the "Y" conformation. Adapted from (Munson & Novick 2006). b) Exocyst complex attached to a vesicle. The Exocyst complex binds to vesicles that have a specific identity given by Rab proteins; Sec5 and Exo84 are effectors and can bind directly to active Ral. Adapted from (Liu & Guo 2012).

One of the next challenges is to understand the molecular mechanisms involved in exocyst specificity: which proteins are upstream and downstream and how can they define where to target a given vesicle. The assembly of the exocyst in a vesicle and the regulation of its traffic and tethering is triggered spatially and temporally by its effectors, but one of the main questions was, what recruits exocyst to the sites of exocytosis. Studies in yeast show that the exocyst subunits Exo70 and Sec3 need to be phosphorylated to bind to phosphatidylinositol (4,5)-bisphosphate (PIP2), which promotes its interaction with the plasma membrane, allowing the specific recruitment to the sites of exocytosis. Exo70 has the capacity to directly associate with phospholipids in the plasma membrane marking the place for vesicle tethering and fusion. Any mutations in these two subunits unable the anchoring of exocyst to the membrane (Finger et al. 1998; He et al. 2007). This model implies that at least two sub-complexes independently exist prior to the formation of the full complex, one at the plasma membrane and one on the secretory vesicle (Matern et al. 2001). The member of exocyst, Sec6 has also been shown to play a role in tethering, by interacting with SNAREs after the binding of the complex to the plasma membrane (Shen et al. 2013). In mammalian cells, Sec6 and Sec8 components of the exocyst have been localized to lateral plasma membranes near the tight junctions of polarized epithelial cells (Grindstaff et al. 1998).

The activity of the Exocyst can also be regulated by the interactions with small GTPases. In yeast, it has been described that the subunit Sec15 can interact with Rab protein Sec4, that when activated recruits exocyst to secretory vesicles (Guo et al. 1999; Wu & Guo 2015). The same interaction was verified in *Drosophila* where Sec15 has been shown to interact with Rab3, Rab8, Rab11 and Rab27 to regulate exocytic trafficking (Wu et al. 2005; Wu & Guo 2015). Rho GTPases can interact with exocyst via Sec3 and Exo70. Cdc42 can interact with Sec3, and it was described to have implications in secretion and in mammalian cells, together with exocyst, to regulate phagocytosis (Mohammadi & Isberg 2013). Interactions with Rho3 GTPase and Exo70 have been implicated in the

incorporation of Glut4 to the plasma membrane in adipocytes in response to insulin stimulation (Wu & Guo 2015).

Ral GTPases can interact with exocyst via Sec5 and Exo84, and these interactions have been implicated in many processes like cell migration, autophagy, neurogenesis and cancer (Moskalenko et al. 2002; Novick & Guo 2002).

From Metazoan, to Fungi, Plantae, all the way to Animals, the exocyst participates in many processes, and mutations in this complex or components of this complex generates a diversity of problems. In Fungi, problems with exocyst lead to problems with cell tip growing, hormone release and affected virulence. In Plantae, mutations in exocyst impaired the guide of root growth and with pollen tubes, mutants have immune responses impaired and interactions with pathogens seemed to target exocyst complex of the host, blocking its effect (Martin-Urdiroz et al. 2016). In Animals, as we described here, the exocyst participates in many trafficking processes and mutations in any subunit lead to many different phenotypes and disorders. In Figure 1.6 are described some of the functions of exocyst in animals and the related phenotype when the complex is impaired. During mice embryogenesis, knockout of Sec3 and Sec8 is lethal from the blastocyst stage and gastrula, respectively (Mizuno et al. 2015; Friedrich et al. 1997). During development, the exocyst is enriched in the placenta and it was suggested that alterations in exocyst function may be associated with preclampsia, which is a trophoblastic condition that leads to poor placental vascularization (Martin-Urdiroz et al. 2016).

In neurons, that are highly polarized cells, the exocyst is present in growth cones, in postsynaptic density regions, axons, and in dendritic branches. At synapses, a specialized form of exocytosis, called synaptic transmission, is surprisingly independent of the exocyst complex (Murthy et al. 2003). However, there is evidence that the exocyst is necessary for neurite growth and the complex Sec6/8 is important for formation of synapses, and is highly expressed in regions undergoing neurite outgrowth and synaptogenesis (Hazuka et al. 1999).

Recent studies associate the exocyst with cancer, in a way that the exocyst is responsible for the formation of filopodia; and invasive cancer cells are characterized by an enormous abundance of filopodia that extend from the cell edge with the principal objective of cell adhesion and migration in an invasive way (Martin-Urdiroz et al. 2016; Tanaka et al. 2016).

One of the most studied effector and promoter of the exocyst assembly is Ral GTPase. It has the capacity to directly bind to two of the exocyst subunits promoting the assembly of the complex and the recruitment of exocyst to sites of higher membrane traffic (Chen et al. 2011).

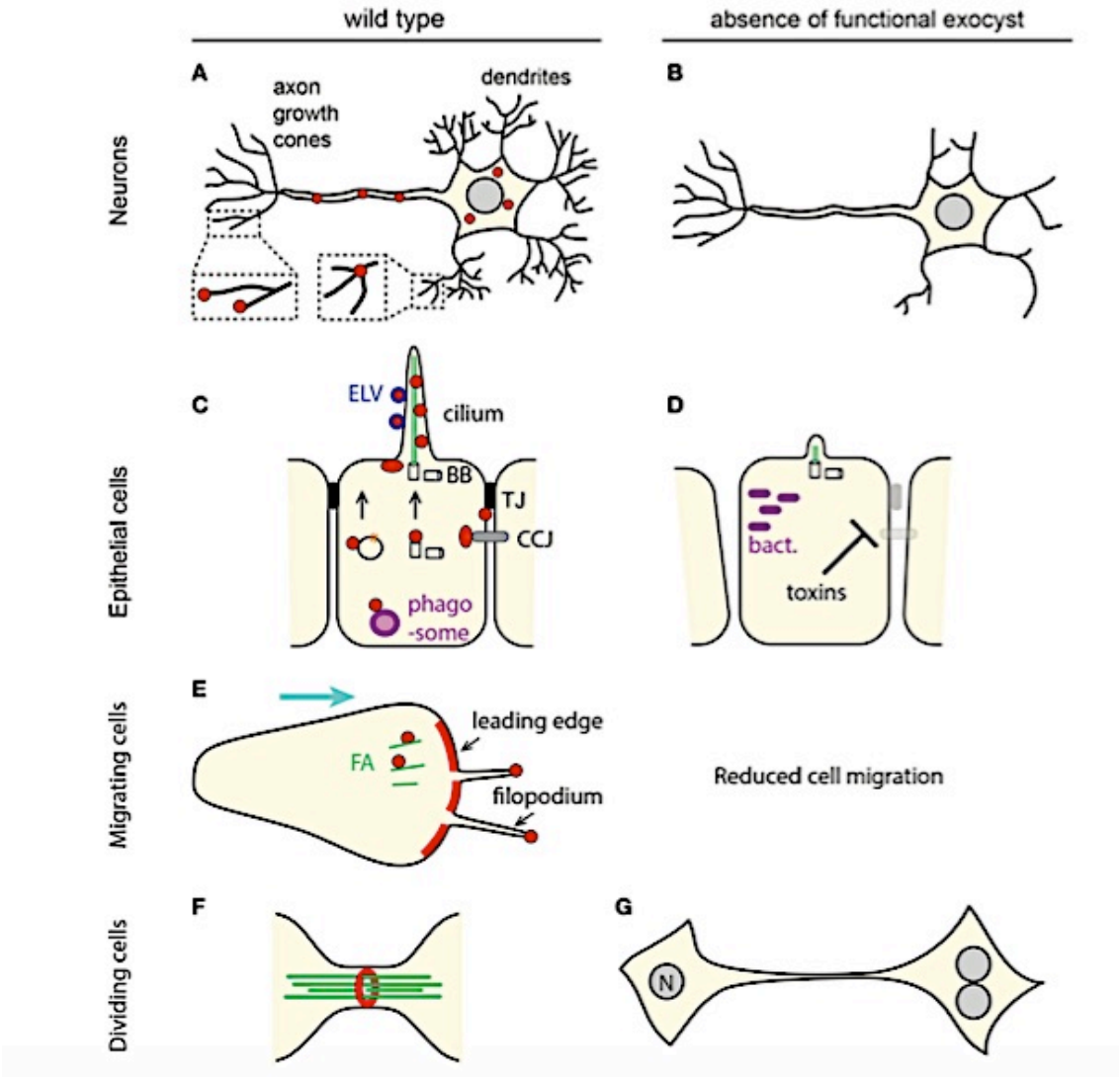


Figure 1.6. Roles of exocyst complex in animals. (A,C,E,F) In this four conditions (**A**, neurites; **C**, cilia; **E**, filopodia and **F**, dividing cells) exocyst is represented in red and as a complex not discriminating any subunit. **(A)** In neurons exocyst can accumulate in dendritic branches, axons and growth cones. **(C,E)** Exocyst is found in tight junctions, and associated with the trafficking of vesicles carrying cell surface receptors. **(F)** In dividing cells exocyst can organize as a ring. **(B,D,G)** Phenotypes associated with the loss of function of exocyst in cells. Cell polarity and tissues integrity are compromised and the protrusions necessary for cell function and migration didn't form. Adapted from (Martin-Urdiroz et al. 2016)

1.3.4. Ral GTPase and the *Exocyst complex*

Ral protein is a small GTPase from the Ras branch superfamily, and like other GTPases is important in the regulation of the transport of vesicles and membranes. Ral was shown to be present at the plasma membrane, in secretory granules and in synaptic vesicle compartments (Shirakawa & Horiuchi 2015; Takai et al. 2001; Moskalenko et al. 2002; Mark et al. 1996). In mammals, Ral protein has two isoforms, RalA and RalB, that share 82% of homology between them. In invertebrates like *Drosophila* or *C. elegans* there is only one Ral gene that is more similar to the RalA isoform. In yeast Ral gene orthologous are not present indicating that the Ral gene emerged during evolution in

multicellular organisms (Shirakawa & Horiuchi 2015; van Dam & Robinson 2006). Plants also lack Ral and, thus far, it is a small GTPase that is only present in metazoans, which coincide with organisms that have a nervous system, which is the tissue where Ral is found in higher abundance (Heider & Munson 2012).

Like other small GTPases, Ral has two inter-convertible forms - an active form (GTP-bound form) and an inactive form (GDP-bound form) - that are regulated by Ral-GEFs and GAPs and can interact with a wide range of effectors, initiating downstream responses and regulate many pathways, like membrane trafficking, actin cytoskeletal reorganization, transcription and kinase cascade activation (Shirakawa & Horiuchi 2015). Ral GTPase is downstream of Ras proteins, which itself receives many upstream signals and can activate a GEF and Ral-GDP dissociation stimulator (Ral-GDS) that specifically delivers Ral proteins to membranes (Feig 2003). Besides being regulated by GEFs and GAPs, Ral can be directly activated in Ca^{2+}/Calmodulin-dependent manner that is not dependent on Ras activation (Sidhu et al. 2005; Hofer et al. 1998). Calmodulin protein is a ubiquitous and conserved Ca^{2+} sensor that can translate calcium signals into a variety of responses. When intracellular calcium increases, calmodulin suffers conformational changes that results in its binding to its target proteins triggering many downstream cascades (Wang & Roufogalis 1999). Ral proteins have binding domains for Ral-GEFs and Calmodulin, as showed in Figure 1.7 that activate Ral and initiate downstream signalling events (van Dam & Robinson 2006).

One of the most studied effectors of Ral is the exocyst complex. Ral has the capacity to bind directly the exocyst subunits Sec5 and Exo84, that compete for Ral due to the overlap of the binding sites, forming a complex capable of regulating many exocytic pathways and cell motility (Sugihara et al. 2002; Moskalenko et al. 2002; Shirakawa & Horiuchi 2015). It has been shown that when active Ral binds to Sec5 and Exo84, it promotes Exocyst assembly, marking the site of exocytosis at the plasma membrane (Moskalenko et al. 2003).

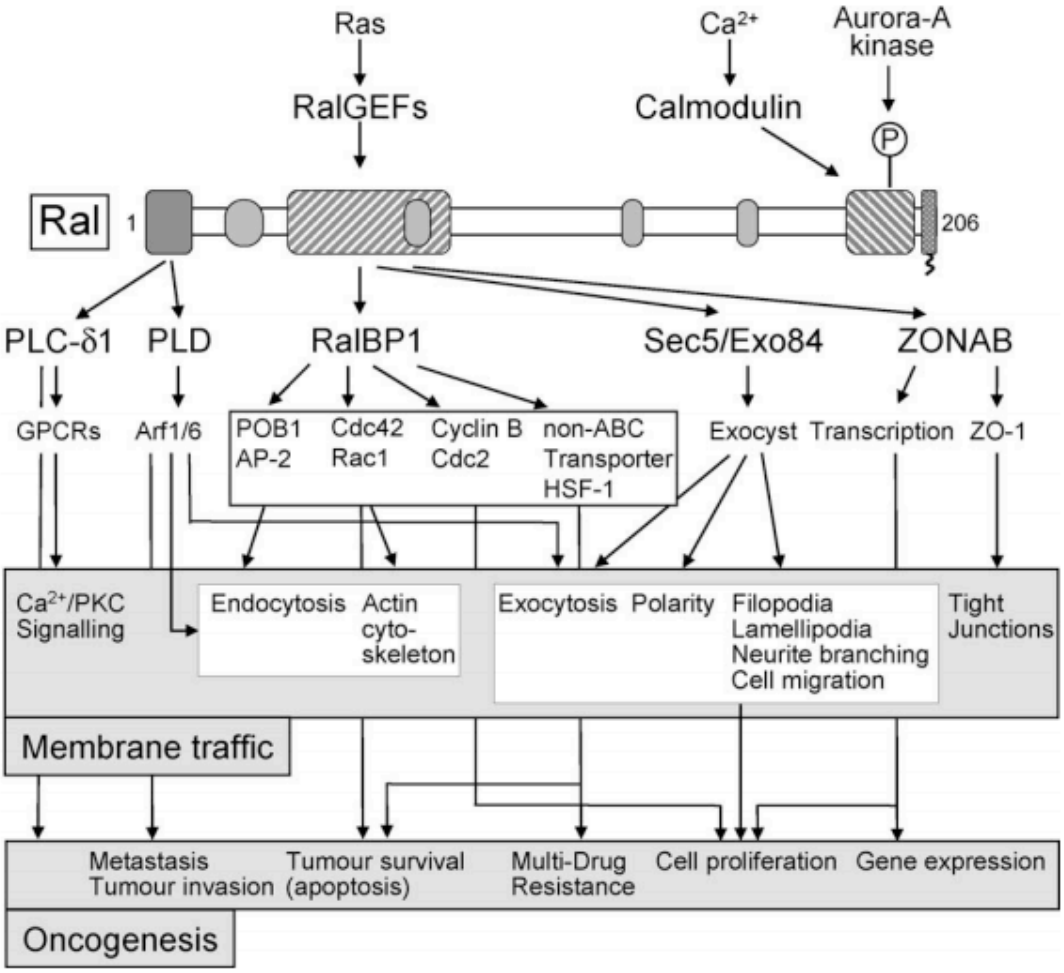


Figure 1.7. Schematic view of Ral protein effectors and how Ral can be implicated in many cellular processes. Ral can be activated by Ral-GEFs, Calmodulin and Aurora-A kinase, represented in the top part of the figure. When activated, Ral can bind to many effectors like, PLC-δ1 (phospholipase C-δ1), PLD (phospholipase D), RalBP1, members of exocyst Sec5/Exo84 and ZONAB. This protein plays different roles in intracellular traffic events and in cell transformation and tumorigenesis. Adapted from (van Dam & Robinson 2006).

Together Ral and the Exocyst complex have the capacity to activate many biological cascades and regulate a variety of processes as it is showed in Figure 1.8. Defects in Ral/Exocyst complex have been associated with a variety of phenotypes, such as, Ral loss of function being associated with partial disassembly of Exocyst, an effect that is similar with loss of Sec4p function in yeast (Guo et al. 1999). In the regulation of exocytosis, activated Ral and the inhibition of Ral function blocked the stimulated release of the human growth hormone by neuroendocrine PC12 cell line (Moskalenko et al. 2002). In neurosecretion, another study showed that a dominant-negative form of Ral diminished the releasable pool of synaptic vesicles docked at the plasma membrane and that RalA binds directly to Sec6/8 complex to target that vesicles to the specific docking sites (Polzin et al. 2002). In summary,

Ral/exocyst signalling is involved in a plethora of events, some of which may be required to regulate neuronal growth and plasticity.

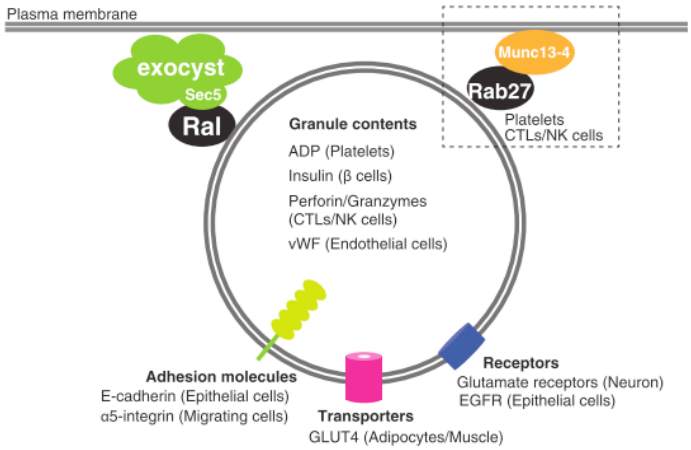


Figure 1.8. Schematic representation of the interactions of Ral/Exocyst complex and granule vesicles. When activated Ral binds do Sec5 promote the assembly of the exocyst complex and together the complex bind to exocytic vesicles. During exocytosis, the content of vesicles is released and exposed to the plasma membrane. The complex plays a role in the release of diverse adhesion molecules, transporters and receptors. In haematopoietic cells, Ral and Exocyst pathways function in concert with Ca^{2+}- regulated Rab27-Munc13-4 pathway to regulate exocytosis. Adapted from (Shirakawa & Horiuchi 2015).

1.3.4.1. Ral/Exocyst complex modulates postsynaptic growth

One of the most well-characterized interactions with exocyst complex is, as described before, the interaction between Ral and Exocyst, that promotes a variety of traffic events in cells (van Dam & Robinson 2006; Sugihara et al. 2002; Moskalenko et al. 2002).

The exocytic trafficking from recycling compartments contributes to dendritic spine growth in response to activity, but this process also requires membrane addition (Park et al. 2006). Therefore, it is possible that the exocyst may be important for synaptic growth and plasticity due to its involvement in the regulation and tethering, docking, and fusion of membrane vesicles to places of growth. In fact, some neuronal functions have been attributed to the exocyst, such as its involvement in neurite outgrowth, in postsynaptic membrane growth, addition of glutamate receptors and maturation of photoreceptors (Gerges et al. 2006; Teodoro et al. 2013).

The exocyst complex distribution in cells is very dynamic and its localization is highly dependent on events that are temporally and spatially regulated. As mentioned before the exocyst can interact with Ral, by binding directly to Sec5 and Exo84 (Moskalenko et al. 2003; Wang et al. 2004). Ral and exocyst are both found in the nervous system (Peng et al. 2004), and since Ral can be activated by Ca^{2+}, the depolarization of membrane in response to synaptic activity can result in the activation of Ral/Exocyst complex. In a previous study, Teodoro *et. al*, took advantage of Ral/exocyst being conserved in *Drosophila*, and studied how activity and the Ral/exocyst pathway can modulate the growth of the postsynpase of *Drosophila* neuromuscular junction (NMJ). Postsynaptic growth in

Drosophila NMJ is dependent on membrane addition, because it is a membranar structure that grows in a activity-dependent manner (Teodoro et al. 2013). The proposed model of the system is described in Figure 1.9.

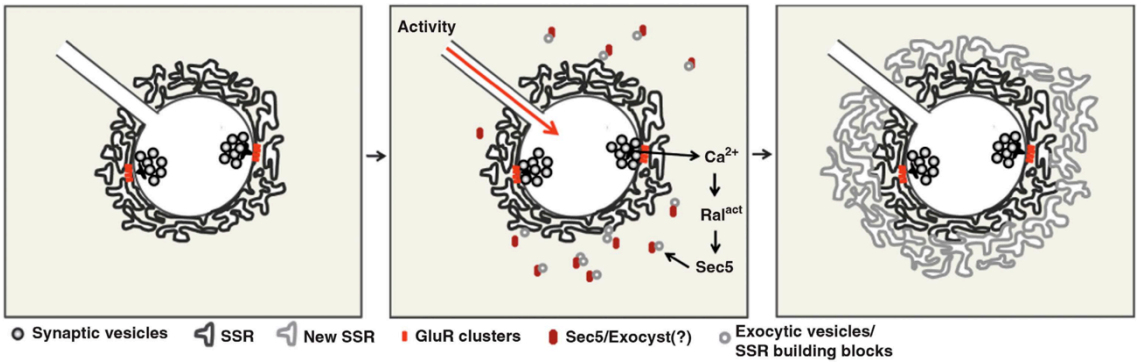


Figure 1.9. Model of Ral and Exocyst involvement in growth of postsynpase in *Drosophila* NMJ. Neuronal activity triggers Ca^{2+} entry into the muscle through activation of postsynaptic glutamate receptors and depolarization of the muscle. The increased cytosolic Ca^{2+} activates Ral and the interaction of Ral with Sec5 causes exocyst-associated membrane vesicles to translocate to and fuse at the postsynapse. The addition of this membrane expands the folds of the SSR around each activated bouton. From Teodoro *et al*, 2013

In this study Rita Teodoro and her collaborators demonstrated that the presence of Ral in the muscle is crucial for these events to occur, and the expression of active Ral postsynaptically induces the recruitment of Sec5 to the NMJ, which leads to a bigger SSR, like it is showed in Figure 1.10 (Teodoro et al. 2013).

For the growth of the SSR to occur, vesicular membrane traffic is involved and required. To have a bigger SSR there is a higher demand of trafficking of vesicles to the SSR region to accommodate the extra growth. One of remaining question is, **where are these vesicles coming from and what is their identity?**

A)

B)

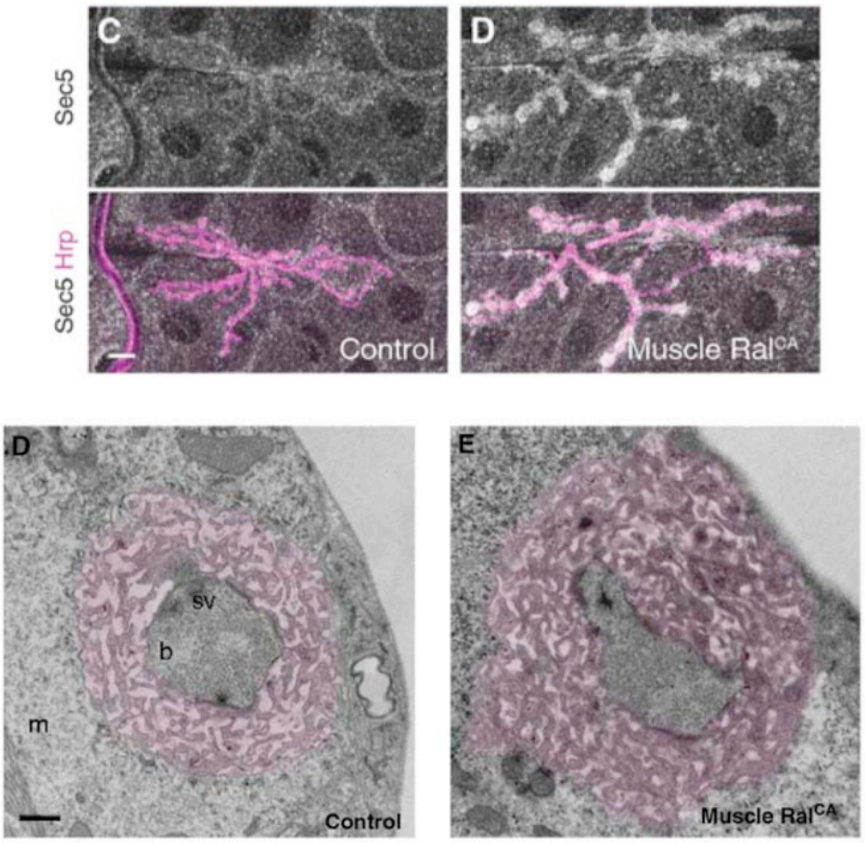


Figure 1.10. A) Constitutively active(CA) Ral in the muscle recruits Sec5 to the NMJ, B) promoting SSR growth. In image C Sec5 distribution in wild type animals is shown. In D it is shown that RalCA expressed in muscle can recruit Sec5 to the NMJ. **B) Effects of Ral expression in postsynaptic structure of NMJ.** Electron microscopy images showing the muscle (m), the synaptic bouton (b) and clusters of synaptic vesicles (sv). The expression of active Ral in the muscle leads to a bigger postsynaptic structure. Adapted from (Teodoro et al. 2013).

Throughout this work we will focus on the SSR of *Drosophila* NMJ to understand what are the mechanisms regulating postsynaptic membrane growth and what are the pathways that regulate it. Since most of the vesicles have a specific small GTPase identity, we will look to that specific identity and try to understand which are the membranous organelles/compartments that are involved in the growth of SSR at the NMJ.

1.4. Drosophila as a model system

Drosophila melanogaster, also known as the fruit or vinegar fly, started to be used in research in the early 1900s by Thomas Morgan and his collaborators. It is one of the most well-studied organisms in biology, having contributed greatly for the research in genetics, development biology and neuroscience. Nowadays, *Drosophila* is considered a great model to study several aspects of cellular biology mostly because it has similar development mechanisms as higher eukaryotes (Celniker 2000;

Demerec & Kaufman 1996). In fact, the genome of fruit fly is predicted to have approximately 75% of homology to known genes related to human diseases (Reiter et al. 2001).

Drosophila is an inexpensive animal to maintain, with a short life cycle, which facilitates experiments and manipulations. Additionally, observations of cells and tissues are relatively simple due to low complexity and size. There are also big resources of genetic tools available and a big research community of *Drosophila* that is happy to share reagents.

1.4.1. Drosophila life cycle

One of the biggest advantages of working with *Drosophila* is its life cycle that is approximately 10 days at 25ºC. When kept at 18ºC, developmental time doubles. *Drosophila* is a holometabolous insect, which means that it undergoes a four-stage life cycle: egg, larva, pupa and adult fly, like it is shown in Figure 1.11. Once fertilized, females can store the sperm and lay hundreds of eggs. At 25ºC, the embryonic development takes approximately 21 hours, and after this period embryo hatches as a larva. The larval stage is divided in three phases called instars, where the larva grows from 1st to 3rd instar. After the 3rd instar stage, larva enter in pupariation where it becomes an immotile pupa, in contrast with what is verified in the larva stage, where they explore and search for food actively. In the pupal stage metamorphosis takes place, degenerating all organs of the larva, and the adult flies emerge from the pupal case after ten days (Demerec & Kaufman 1996).

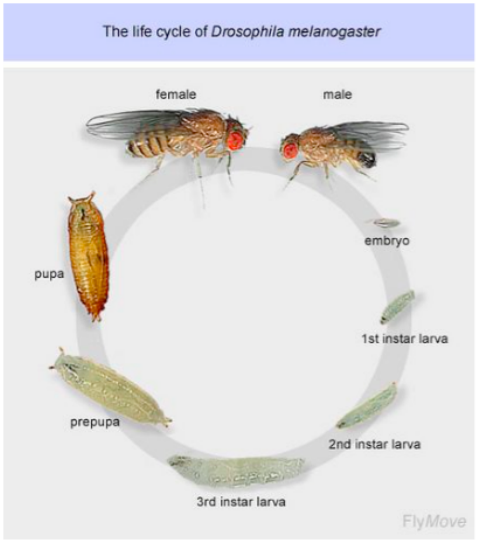


Figure 1.11. *Drosophila* life cycle. Adapted from (Weigmann et al. 2003)

1.4.2. Neuromuscular Junctions in Drosophila

In *Drosophila,* the neuronal muscular junctions (NMJ) are a well established model system to study synaptic development and function. The motor neurons that innervate the muscles are excitatory and glutamatergic, which makes this synapse an ideal model to study mammalian central glutamatergic neurons, given that the same cellular and molecular components found in invertebrate

and vertebrate excitatory synapses (Collins & DiAntonio 2007). The larval NMJ synapses use ionotropic glutamate receptors that are homologous to AMPA-Type glutamate receptors in the mammalian brain (Menon et al. 2013).

Wild type *Drosophila* larvae have a stereotyped body wall muscle pattern that is organized in repeating segments that are bilaterally symmetric. Each hemisegment has 30 individually identifiable muscles that are innervated by about 40 motor neurons (Gramates & Budnik 1999). During embryogenesis of *Drosophila* NMJ, motor neurons extend their axons into the musculature, being each motor axon genetically guided to the path of a specific muscle fiber or group of fibers. After the axonal growth cones established the contact with the muscle, embryonic synapses are made and continually modified during development. A coordinated maturation of pre-(neuronal) and potsynaptic (muscle) has to occur in order to generate functional synpases. Initially each NMJ has a few synaptic boutons, that are oval-shaped structures embedded in the muscle, that eventually will be surrounded by an infolded membranar structure called Subsynaptic Reticulum (SSR). The SSR contains neurotransmitter receptors, scaffolding proteins and neurotransmitter signalling complexes (Oh & Robinson 2012; Gramates & Budnik 1999; Ruiz-Cañada & Budnik 2006; Menon et al. 2013). In this work we are focused on the postsynapse of the NMJ, where we can find postsynaptic glutamatergic receptors and Discs large (Dlg). Dlg is the *Drosophila* ortholog of the mammalian scaffolding protein PSD-95 (Menon et al. 2013). There are two specific known postsynaptic membrane markers in *Drosophila* Dlg and Syndapin (Kumar et al. 2009). Dlg is a tumor suppressor gene that encodes a protein necessary for normal growth in epithelial cells and brain tissue. It shares a high sequence identity to the mammalian synaptic proteins PDS-95 and SAP-102. It was describe that, mutations in Dlg lead to problems with the postsynapse, suggesting that Dlg is necessary for the normal growth of the SSR (Lahey et al. 1994). Syndapin, or Synaptic dynamin associated proteins, is a protein that belongs to the F-BAR domain protein family, whose predicted functions in membrane tubulation remain poorly studied in vivo (Utani 2010), but is conserved from insects to mammals (Kessels & Qualmann 2002). In *Drosophila*, Syndapin is predominantly localized at NMJ in 3rd instar larvae, it promotes formation of SSR, by a mechanism that requires the F-BAR domain (Utani 2010). In mammalian neurons, Syndapin is localized in postsynaptic dendritic spines where it regulates the endocytosis of NMDA receptors (Pérez-Otaño et al. 2006).

The growth and development of *Drosophila* NMJs are highly stereotyped, predictable and reproducible. Importantly, the NMJ is structurally and functionally adaptable making it an excellent model system to study the effect of genetic and environmental perturbations on synapse development (Oh & Robinson 2012).

1.4.3. Genetic tools

In the past century, an incredible variety of genetic tools has become available for studying *Drosophila* using several techniques. Here we review some tools that were used in this project.

1.4.3.1. UAS/Gal4 System

One of the most used genetic tools that allows gene manipulation in *Drosophila* is the GAL4-UAS system, that is used to do specific spatial and temporal regulation of gene expression. This system was developed by Andrea Brand and Norbert Perrimon using Gal4, a transcription activator factor that was found in *Sacharomyces cerevisiae.* GAL4 can regulate gene expression in *Drosophila* by binding directly to Upstream Activating Sequences (UAS), that are enhancer elements near to gene of interest (Brand & Perrimon 1993). This is a bipartite system, where with a simple fly cross, the progeny will expresse the target gene in a pattern dictated by the expression of GAL4. In the absence of GAL4 the target gene is silent. This way we have flies with the GAL4 "driver" to a specific tissue, for example, and flies with the transgene of interest under the control of UAS present upstream of the gene of interest, that will lead to a progeny that have the transgene expressed only were GAL4 is present, like is showed in Figure 1.12 (Caygill & Brand 2016; Brand & Perrimon 1993).

This system can be regulate also by temperature, where the minimal GAL4 activity is at 16ºC and at 29ºC the activity of GAL4 is a balance between the maximal activity and the minimal effects on fertility and viability due the to high temperature (Duffy 2002).

One of the biggest advantages of this system is the thousands of GAL4 lines available and others like, ability to label cells, make targeted mutations, express or knockdown genes of interest *in vivo* (Caygill & Brand 2016).

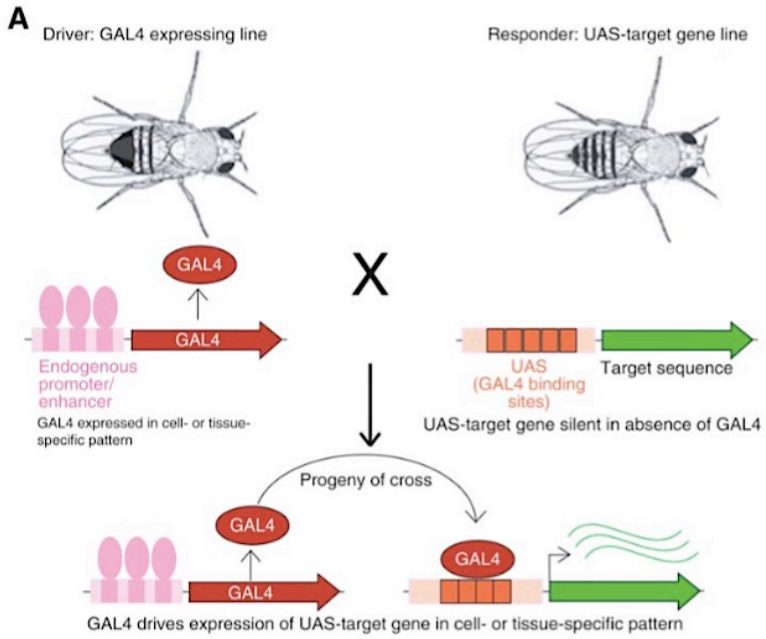


Figure 1.12. Schematic representation of the UAS/Gal4-based transgene expression. Adapted from (Elliott & Brand 2008; Weigmann et al. 2003)**.**

1.4.3.2. RNA interference

RNA interference (RNAi) is a technique for gene silencing in the organism and in cultured cells, and it was first described in *C. elegans* (Fire et al. 1998). This method relies on sequence homology between double-stranded RNA (dsRNA) and the target mRNA. In *Drosophila*, the dsRNA is expressed in the target cells or tissues as hairpin RNA taking the advantage of the GAL4/UAS system that can control the expression spatially and temporally (Yamamoto-Hino & Goto 2013).

The RNAi construct contains an inverted repeat sequence, with homology to the target gene, that allows the formation of the hairpin structure upon transcription, and it is cleaved by an endogenous enzyme called Dicer. Dicer cuts the hairpin in small fragments that will act as templates for the RNA-induced silencing complex (RISC) that will recognize specifically the mRNA and cleave it (Yamamoto-Hino & Goto 2013).

In *Drosophila*, the use of RNAi strategies became a powerful tool in alternative to the use of mutant alleles. With the key advantage that flies carrying UAS-RNAi constructs allow the targeted knock-down of specific genes in a specific tissue or set of cells, and sometimes at distinct stages of development.

1.5. Aims of the work

The aim of our work is to characterize the molecular mechanisms that convert synaptic activity into postsynaptic membrane growth in a Ral/Exocyst-dependent manner. We focused on a novel pathway that regulates neuronal morphology in response to activity through the engagement of Ral and the Exocyst complex in the regulation of membrane growth at the synapse in response to neuronal activity. Since we know that Ral and Exocyst are required to form a complex which recruits membrane vesicles that allow the SSR to grow, and knowing that Rab GTPases are traffic regulators of many biological processes, we screened the Rab GTPases family in *Drosophila* (28 Rabs know) to better understand the nature of the vesicles that contribute to SSR growth. Understanding which subset of Rabs mediate structural plasticity gives us clues about the vesicle content, the composition of the SSR and the receptors and effectors on the postsynaptic membrane of the NMJ. We expect to identify the pathway that leads to the growth of postsynaptic structure in glutamatergic neurons of *Drosophila*, which will lead to a better understanding of molecular pathways that regulate neuronal growth in excitatory synapses in vertebrates.

Chapter 2. Materials and Methods

2.1 Fly stocks and husbandry

In this work all fly stocks were maintained at room temperature and kept in vials containing fly food (a mixture of water, agar, sugar, corn meal, yeast and fungicides). When performing experiments and crosses the animals were maintained at 25ºC or 30ºC in an appropriate atmosphere with controlled humidity. To step up the crosses, female virgins were collected as described by Ashburner in (Ashburner & Roote 2007).

The *Drosophila* stocks used are described in Table 2.1 The stocks were obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/) or generated in our laboratory.

Table 2.1. Detailed list of Drosophila stocks used in this work.

Name	*Genotype*	*From*
Rab1 IR	y^1 sc* v^1; P{TRiP.HMS01148}attP2	BDSC #34670
Rab2 IR	y^1 sc* v^1; P{TRiP.HMS01271}attP2	BDSC #34922
Rab3 IR	y^1 sc* v^1; P{TRiP.HMS01131}attP2	BDSC #34655
Rab4 IR	y^1 sc* v^1; P{TRiP.HMS01100}attP2	BDSC #33757
Rab5 IR	y^1 sc* v^1; P{TRiP.HMS00147}attP2	BDSC #34832
Rab6 IR	y^1 sc* v^1; P{TRiP.HMS01486}attP2	BDSC #35744
Rab7 IR	y^1 v^1; P{TRiP.JF02377}attP2	BDSC #27051
Rab8 IR	y^1 sc* v^1; P{TRiP.HMS01363}attP2	BDSC #34373
Rab9 IR	y^1 sc* v^1; P{TRiP.HMS02635}attP40	BDSC #42942
Rab10 IR	y^1 v^1; P{TRiP.JF02058}attP2	BDSC #26289
Rab11 IR	y^1 v^1; P{TRiP.JF02812}attP2	BDSC #27730
Rab14 IR	y^1 sc* v^1; P{TRiP.HMS01130}attP2	BDSC #34654
Rab18 IR	y^1 sc* v^1; P{TRiP.HMS01214}attP2	BDSC #34734
Rab19 IR	y^1 sc* v^1; P{TRiP.HMS00592}attP2	BDSC #34607
Rab21 IR	y^1 v^1; P{TRiP.JF03338}attP2	BDSC #29403
Rab23 IR	y^1 v^1; P{TRiP.JF02859}attP2	BDSC #28025
Rab26 IR	y^1 v^1; P{TRiP.JF01684}attP2	BDSC #31177
Rab27 IR	y^1 sc* v^1; P{TRiP.HMS01523}attP2	BDSC #35774
Rab30 IR	y^1 v^1; P{TRiP.JF01593}attP2	BDSC #31120
Rab32 IR	y^1 v^1; P{TRiP.JF02836}attP2	BDSC #28002
Rab35 IR	y^1 v^1; P{TRiP.JF02978}attP2	BDSC #28342
Rab39 IR	y^1 v^1; P{TRiP.JF01973}attP2	BDSC #25953
Rab40 IR	y^1 v^1; P{TRiP.JF03258}attP2	BDSC #29579
RabX1 IR	y^1 v^1; P{TRiP.JF02868}attP2	BDSC #28033
RabX2 IR	y^1 sc* v^1; P{TRiP.HMS00351}attP2	BDSC #32360
RabX4 IR	y^1 sc* v^1; P{TRiP.HMS02787}attP40	BDSC #44070
RabX5 IR	y^1 v^1; P{TRiP.JF02880}attP2	BDSC #28045
RabX6 IR	y^1 v^1; P{TRiP.JF02050}attP2	BDSC #26281
Rab1 YFP	w^{1118}; TI{TI}$Rab1^{EYFP}$	BDSC #62539
Rab2 YFP	w^{1118}; TI{TI}$Rab2^{EYFP}$/CyO	BDSC #62540
Rab3 YFP	W^{1118};TI{TI}$Rab3^{EYFP}$	BDSC #62541
Rab4 YFP	y^1 w^{1118}; TI{TI}$Rab4^{EYFP}$	BDSC #62542
Rab5 YFP	w^{1118}; TI{TI}$Rab5^{EYFP}$	BDSC #62543
Rab6 YFP	w^{1118}; TI{TI}$Rab6^{EYFP}$	BDSC #62544
Rab7 YFP	w^{1118}; TI{TI}$Rab7^{EYFP}$	BDSC #62545
Rab8 YFP	w^{1118}; TI{TI}$Rab8^{EYFP}$	BDSC #62546
Rab9 YFP	w^{1118}; TI{TI}$Rab9^{EYFP}$	BDSC #62547
Rab10 YFP	w^{1118} TI{TI}$Rab10^{EYFP}$	BDSC #62548
Rab11 YFP	w^{1118}; TI{TI}$Rab11^{EYFP}$	BDSC #62549
Rab14 YFP	w^{1118}; TI{TI}$Rab14^{EYFP}$/CyO, P{hs-hid}4	BDSC #62550
Rab18 YFP	$y^1w^{1118}y^1$TI{TI}$Rab18^{EYFP}$	BDSC #62551
Rab19 YFP	w^{1118}; TI{TI}$Rab19^{EYFP}$	BDSC #62552
Rab21 YFP	y^1 w^{1118} TI{TI}$Rab21^{EYFP}$	BDSC #62553
Rab23 YFP	y^1 w^{1118}; TI{TI}$Rab23^{EYFP}$	BDSC #62554
Rab26 YFP	y^1 w^{1118}; TI{TI}$Rab26^{EYFP}$	BDSC #62555
Rab27 YFP	y^1 TI{TI}$Rab27^{EYFP}w^{1118}$	BDSC #62556
Rab30 YFP	w^{1118}; TI{TI}$Rab30^{EYFP}$	BDSC #62557
Rab32 YFP	w^{1118}; TI{TI}$Rab32^{EYFP}$	BDSC #62558

Rab35 YFP	w^{1118} TI{TI}Rab35^{EYFP}	BDSC #62559
Rab 39 YFP	w^{1118} TI{TI}Rab39^{EYFP}	BDSC #62560
Rab40 YFP	y^{1} w^{1118} TI{TI}Rab40^{EYFP}	BDSC #62561
RabX1 YFP	w^{1118}; TI{TI}RabX1^{EYFP}	BDSC #62562
RabX4 YFP	w^{1118}; TI{TI}RabX4^{EYFP}	BDSC #62563
RabX5 YFP	w^{1118}; TI{TI}RabX5^{EYFP}/TM6B, Tb^{1}	BDSC #62564
RabX6 YFP	w^{1118}; TI{TI}RabX6^{EYFP}	BDSC #62565
UAS-GFP.Valium10	$Y^{1}v^{1}$;P{UAS-GFP.VALIUM10}attP2	BDSC #35786
MHCGS RalCA	W1118; MHCGS-Gal4, UAS-RalCA(G20V)	Teodoro Lab
MHCGS RalWT	W1118; MHCGS-Gal4, UAS-RalWT	Teodoro Lab
D2;G14/CAG	UAS-Dicer2; G14-Gal4/CyO, ActGFP	Teodoro Lab
W^{1118}	W[1118]	Teodoro Lab

2.2. Characterization of Rab endogenous distribution

From previous studies, mutations in Sec5 or Ral proteins cause diminished SSR (Teodoro et al. 2013). To evaluate which Rab proteins are expressed in the muscle or at the NMJ, so they can regulate the transport of vesicles to the plasma membrane to support SSR growth, we performed a series of experiments where we evaluated the endogenous distribution of Rabs. To do this, we used a collection of flies where each individual Rab is endogenously tagged with YFP. These YFP stocks were dissected and processed for immunocytochemistry. In order to see if the localization of each Rab was altered in the presence of muscle RalCA or muscle RalWT, we crossed virgin females of the genotype MHCGS-Gal4,UAS-RalCA or MHCGS-Gal4,UAS-RalCA with males of each of RabX-YFP. The F1 larvae were dissected and processed for immunocytochemistry.

2.3. Identification of Rab candidates that contribute to SSR growth

To dissect the pathway that regulates SSR growth in a Ral-dependent manner, we screened all Rab GTPases using a RNAi approach. We reduced the levels of each Rab in the background of muscle RalCA or muscle RalWT, and screened for reduced levels of Sec5 recruitment to the NMJ.

Virgin females of the genotype MHCGS-Gal4,UAS-RalCA or MHCGS-Gal4,UAS-RalCA were crossed with UAS-RabX RNAi flies (experiment performed at 30ºC to enhance the efficiency of RNAi) and F_1 3rd instar larvae were dissected and processed for immunocytochemistry. For these experiments with RNAi the control used was UAS-Valium10 which expresses a GFP under the control of UAS in the Valium10 vector, which is the same vector backbone present in the RNAi lines.

For the candidates identified in the screen, we repeated the experiment three times, and quantified Sec5 recruitment (see bellow).

2.4. Immunocytochemistry assays

2.4.1. Larval dissection and fixation

To collect and dissect 3rd instar larvae, we did crossed in vials that were kept at 25ºC or 30ºC (in the case of RNAi) with standard fly food, the 3rd instar larvae appear after ~4 to 5 days. The 3rd instar larvae were selected and dissected in sylgard (transparent silicone rubber) plates in a drop of Phosphate Buffer Saline (PBS 1x) using forceps (FST Dumont #5 and #3) as described by (Brent et al. 2009). First, we place a pin between the anterior mouthparts and then put the second pin between

the posterior spiracles stretching the animal (dorsal parte up) to maximize the amount of exposed body wall during cutting. Using spring scissors we make a horizontal incision anterior to the posterior pin on the dorsal side of the larvae, after that we put one blade of the scissors in to the incision and make a vertical cut along larvae until the anterior pin. With the forceps we remove the organs pin the flaps of the larvae body wall in a clockwise order making sure to stretch the body wall both vertically and horizontally. The dissected body wall was immediately fixed in a fixative solution of 4% of paraformaldehyde (PFA) in 1x PBS for 20 min at RT. After fixation the larvae were washed with PBT which permeabilizes membranes.

2.4.2. Immunofluorescence protocol

After fixation and permeabilization, larvae were incubated with blocking solution (5% of normal goat serum (NGS) in PBT) for 1h, followed by overnight (ON) incubation with primary antibodies at 4ºC in diluted blocking solution. After that, larvae were rinsed 3×15 min with PBST (PBS1x, 0.3%triton), blocked again and incubated 2h with the secondary antibodies at RT in diluted blocking solution. Larvae were rinsed after secondary antibodies 3 × 15min in PBT and put one by one in Glycerol solution 50% for 10 min. All samples were then mounted in DABCO mounting medium (1,4-Diazabicyclo[2.2.2]octane, Sigma-Aldrich) in microscope slides and stored at 4ºC protected from light. All antibodies used are described in Table 2.2 and Table 2.3. In *Drosophila*, HRP labels a neuronal protein and is therefore used to label the neuronal membrane.

Table 2.2. Primary antibodies used in immunofluorescence assays.

Protein	*Host*	*Dilution*	*Supplier*
Sec5	Mouse	1:35	Rita Teodoro Lab
GFP	Rabbit	1:1000	Life Technologies
Syndapin	Guinea Pig	1:1000	Donated by Vimlesh Kumar

Table 2.3. Secondary Antibodies and dyes used in immunofluorescence assays

Antibody	*Dilution*	*Supplier*
Alexa Fluor A488 anti-rabbit	1:500	Jackson Immuno Research
DL A647, anti-HRP	1:500	Jackson Immuno Research
Alexa Fluor A488 anti-Guinea Pig	1:500	Jackson Immuno Research
Rhodamine Red-X anti-mouse	1:500	Jackson Immuno Research

2.4.3. Image acquisition and Image Analysis

Larval imaging was performed in a Zeiss LSM 710 Confocal Microscope using a 40x water objective and images were analyzed using in FIJI software and Adobe Photoshop.

In order to quantify the recruitment of Rab proteins to the NMJ, we used Sec5 as a readout. At least ten NMJs were analyzed per condition. To quantify Sec5 recruitment we used the HRP channel to outline the NMJ, and expanded it to 2 or 5 interactions (postsynaptic area surrounding the NMJ, corresponding to the SSR). Using this expanded HRP we measured the area, which corresponds to

the “synaptic area”. We also take the intensity of Sec5 in the muscle in a square that is 10 x 10 um^2. Taking the synaptic area, we measure the intensity of Sec5 that is within that area, and subtract the value of Sec5 in the muscle. This value represents Synaptic Sec5 – muscle Sec5 and is a measure of Sec5 enrichment.

To quantify the amount of syndapin present in the NMJ, we use the same procedure, but we don’t take any muscle measurements, given that Syndapin is only localized at the NMJ.

2.5. Activity-dependent recruitment of Sec5 to the NMJ

Because synaptic activity has been shown to induce Sec5 recruitment to the NMJ in a Ral-dependent manner, supposedly contributing to SSR growth in response to synaptic activity, we wanted to test if our Rab candidates are required for this process. To do this, we performed a stimulation assay that mimics synaptic activity, where 3 pulses of high K^+ and high Ca^{2+} (1mM), intercalated with normal K^+ and 0 Ca^{2+} are administered to the dissected larvae. This induces body wall contractions and results in the formation of new synaptic boutons.

For these experiments, we collected virgin females of the genotype UAS-Dicer2; G14-Gal4 (where G14 is a strong muscle driver), and crossed with males of each of the UAS-RabX-RNAi (Rab1, 10 and X4). The F1 3rd instar larvae of the candidate Rabs RNAi were dissected as described before but without taking out the internal organs the stimulation protocol is performed. At this point, the larvae cannot be very stretched to allow for contractions. The protocol starts with 2 minutes of stimulation with a high K^+ solution, followed by 10 minutes of rest with a solution with zero Ca^{2+}, called HL.3.1. The solutions used were adapted from (Feng et al. 2004).These periods of rest and stimulation are repeated 3 times finishing with 15 minutes of rest. During the last rest time, we remove the internal organs and stretch the larvae body wall to complete the dissection and proceed with the immunochemistry assay.

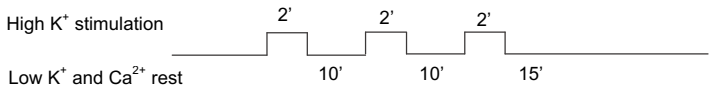


Figure 2.1. Schematic representation of the stimulation paradigm used. 3 stimulations of 3 times 2 minutes, interspaced by 10 minutes. At the end, larvae are fixed and processed for immunocytochemistry.

2.6. Transmission Electron Microscopy

2.6.1. Sample preparation and fixation

For Transmission Electron Microscopy 3rd instar larvae of the Rab candidates were selected and dissected as described before, but in a PHEM buffer, and the fixation was performed with Karnovsky’s Fixative which combines 2% of paraformaldehyde and 2,5% of glutaraldehyde to achieve a more rapid overall penetration of the fixative (Bozzola & Russell 1999). After the overnight fixation, the samples were post-fixed with 1% of osmium and 0,8% of Potassium Ferricyanide, followed by a series of dehydration solutions until a 100% ethanol dehydration. The samples were then put in 1:1 of

ethanol and fresh resin for one hour and after that, 1:2 of ethanol and fresh resin ON. The following day, samples were placed in 100% fresh resin before embedding.

2.6.2. Embedding and sectioning of samples

The samples were embedded in Epon resin in specific molds to perform a flat embedding of the larvae. On top of the molds we put some weight and let the resin solidify 24h at 60ºC. The samples were sectioned with Leica UC7/FC7 Ultramicrotome with a Diatome Diamond Knife with a maximum thickness of 90nm. The specimens are mounted in TEM grids of Copper-Palladium covered with Formvar, which is a polymer much thinner than glass and gives support to the samples in the grids. After mounting, the samples were stored in grids boxes at RT.

2.6.3. Imaging and Image Analysis

The imaging of the samples was done on Hitachi H-7650 TEM and images were analyzed using FIJI software.

Chapter 3. Results and Discussion

The main goal of this work is to understand the cellular and molecular mechanisms by which synaptic activity regulates synaptic growth and plasticity. More specifically we aim to dissect the genetic cascade that converts synaptic activity into postsynaptic membrane growth in a Ral/exocyst-dependent manner, using the *Drosophila* NMJ.

In previous studies Teodoro and collaborators established that SSR growth is dependent on activity and that the recruitment of vesicles to the membrane by the Ral/Exocyst pathway is required for growth (Teodoro et al. 2013). In many types of cells, neurons included, most of the exocytic and endocytic pathways are regulated by small GTPases, like RabGTPases (Tojima & Kamiguchi 2015). Given this, we characterized the distribution of all Rabs at the *Drosophila* NMJ and muscle to see which Rab GTPases are expressed in these tissues. Then, knowing from Teodoro studies that the presence of active Ral in the muscle can alter the distribution of recruited complexes and vesicles to the NMJ, we tested whether expression of Ral wild-type (WT) or Ral constitutively active (CA) in muscle modifies the normal distribution of RabGTPases. These studies were possible due to the existence of an elegant collection of flies that have endogenously tagged each Rab with YFP (Dunst et al. 2015).

To address the question of which Rab(s) proteins are involved in the recruitment of vesicles to the NMJ, we screened by RNAi the 28 Rab proteins of *Drosophila* looking for any significant changes in the recruitment of Sec5. We used Sec5 as readout for exocyst localization, because it has been described to be recruited to the NMJ in response to activity and in the presence of active Ral in the muscle (Teodoro et al. 2013). We expect this recruitment to be consistent and reproducible unless there are mutations in other components of the cascade that would interfere with Sec5 localization. Therefore, the main objective in this part of the study was to detect alterations in Sec5 recruitment, by doing RNAi against Rabs, which could indicate that some Rab or Rabs might be involved in this pathway. Identification of the identity of the vesicles responsible for SSR growth will allow us to dissect the genes required for the signaling cascade triggered by Ca^{2+} activation of Ral, and will complete our understanding of this pathway and the factors that participate in this conserved form of plasticity.

3.1 Characterization of the endogenous distribution of Rab proteins in *Drosophila* larvae

Rab proteins are small GTPases that are master regulators of intracellular membrane traffic. To understand how postsynaptic membrane growth is regulated, we started by characterizing the localization of each Rab GTPase at the *Drosophila* NMJ. In order to describe the distribution of Rab proteins in the *Drosophila* 3rd instar larvae, we used a collection of fly stocks that have an YFP-tag in the endogenous locus of 27 out of 28 Rabs present in the *Drosophila* genome, as schematized in Figure 3.1 (Dunst et al. 2015). Knowing the localization of each Rab GTPase will help us understand and possibly infer about their possible functions. Given that we are interested in the dissection of the molecular pathways that regulate postsynaptic growth, we can also identify, based on localization, Rab candidates that may contribute to this process.

Figure 3.1. Construct of YFP^{MYC} of the Rab alleles. YFP and MYC sequences were inserted into each Rab *locus.*Adapted from (Dunst et al. 2015).

For our analysis, we were interested mostly in Rab GTPases that were expressed postsynaptically and also in neurons. Therefore, we grouped Rabs in three categories: 1) not expressed in muscle, 2) expressed in muscle and 3) expressed at the synapse. With the principal objective of making an overview characterization of Rab expression.

There are few studies that describe the localization of Rabs in *Drosophila* (Chan et al. 2011; Zhang et al. 2007). A recent study characterized neuronally expressed RabGTPases and tried to dissect their possible role in presynaptic function. For this, Chan *et al.*, characterized the distribution of Rabs in the larval and in the adult brain and, surprisingly, the authors found that almost half of the *Drosophila* Rab GTPases are expressed in subsets of neurons in brain, and many of those Rabs encode synaptic proteins. They identified a subset of Rabs that are only expressed in neurons and possibly glia specific in the larva brain (Rab3, Rab19, Rab26, Rab27, Rab32 and RabX4), and with this set of Rabs, they also looked at its distribution at the NMJ. It was possible to identify Rab3, RabX4, Rab26 and Rab19, in presynaptic boutons and by the contrary Rab23 is expressed in NMJ but it looks that is distributed outside of boutons (Chan et al. 2011). A big difference between this study and ours, besides looking at the presynaptic side exclusively, is the fact that they used a Rab-Gal4 crossed with UAS-Rab-WT-YFP: this allowed for Rab expression in the correct tissues but induces over-expression of each of the Rabs. In our experiments, using this newly generated collection of YFP-tagged Rab GTPases, we are able of looking at endogenous levels of expression.

One of the best-characterized neuronal Rab is Rab3, which has a known neuronal distribution that we can confirm in our analysis (Figure 3.2.). Rab3 has been shown to be expressed pre-synaptically and in the active zones in *Drosophila* NMJ and in no other tissue (Junction et al. 2016; Graf et al. 2009), which is exactly what we observe using this EYFP reporter, serving as a positive control.

The characterization of Rabs in *Drosophila* 3rd instar is described in Figure 3.2. and the phenotypes observed were consistent in every animal and experiment, we normally used 6 animals per genotype.

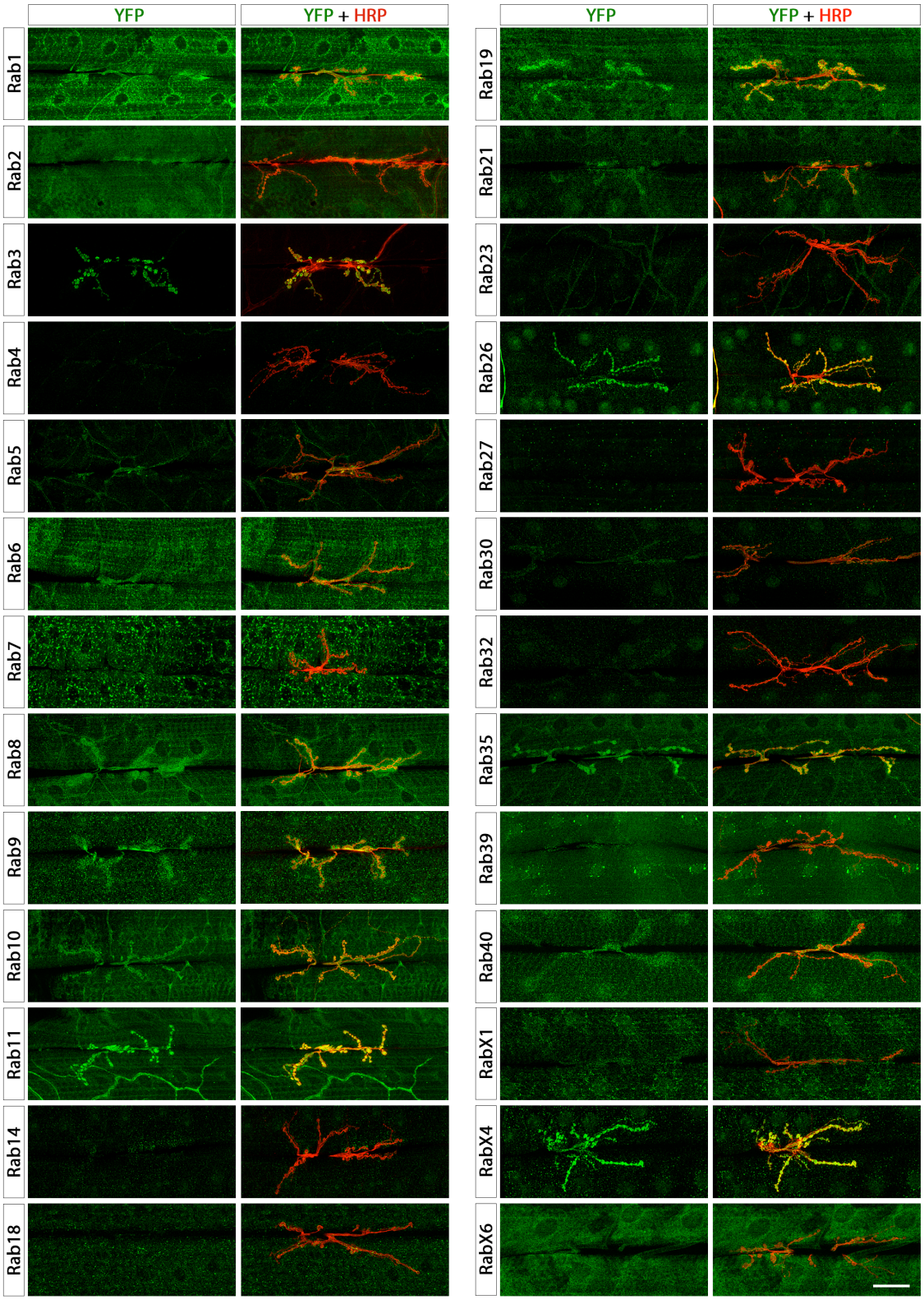


Figure 3.2. Characterization of RabGTPases distribution in *Drosophila* NMJ. The panel show the distribution of RabGTPases in NMJ of muscles 6/7 of 3rd instar larvae, segment A2 or A3. For all images, scale bar 10 μm.

Most of the Rabs are present in the muscles of *Drosophila*, some are uniformly distributed, while others have a perinuclear distribution. Rab1, Rab2, Rab6, Rab7, Rab14, Rab18, Rab21, Rab26, Rab27, Rab30, Rab32, Rab35, Rab39, Rab40, RabX1, RabX4, RabX6 have a ubiquitous distribution in the muscle of the larvae. Some Rabs are more expressed than others, which is the case of Rab14, Rab18, Rab27, Rab30 and Rab32 that have much less expression in muscles when in comparison to other Rabs. Interestingly, we were not able to see expression of Rab32 in the NMJ like it was described previously by Chan *et al* (Chan et al. 2011).

Rab3, Rab8, Rab9, Rab10, Rab11, Rab19, Rab26, Rab35 and RabX4 have a synaptic distribution. Almost all Rabs that are synaptic are also expressed in muscle, except Rab3, that is exclusively synaptic, as described by Junction *et al* and Graf *et al*. Rab8 by the contrary looks postsynaptic, but more co-localization studies have to be performed to confirm. Rab10, Rab11 and Rab35 are synaptic but are also expressed in the entire muscle. Rab26 is mostly synaptic and perinuclear and RabX4 has more intensity in the synapse but also has a punctated distribution in muscle. When looking to the set of the Rabs that have a muscle distribution, we can see a variety of intensities and some are more concentrated around the nucleus of the muscle, like Rab30, Rab32 and Rab39.

In Figure 3.2 RabX2 is not present because the construct wasn't available in Rab EYFP-tag collection (Dunst et al. 2015) and RabX5 wasn't yet analyzed.

This characterization of the endogenous distribution is a powerful tool to identify and exclude candidates that might be related to our question of which Rabs interfere with SSR growth. At the end of these experiments our attention turned mostly to the Rabs that are synaptic, and if these Rabs change their distribution in the presence of higher levels of active Ral, they would be good candidates to mediate Ral/Exocyst-dependent growth of the SSR. In another words, these Rabs could be upstream regulators of this cascade. However, if no change in distribution is observed, one cannot discard the Rabs since a steady-state may have been achieved that is indistinguishable from the normal situation.

3.1.1. Characterization of the distribution of Rab proteins in the presence of Ral CA and RalWt in the muscle

From previous studies, it is described that when constitutively active or wild type Ral is over-expressed in the muscle, there is recruitment of the Exocyst to the synaptic region, which leads to a larger postsynaptic membrane structure – the SSR. While the recruitment of Sec5 is observed when both RalWT and RalCA are expressed, there is significantly more in the case of RalCA (Teodoro et al. 2013). In order to understand if the distribution of Rab proteins changes in the presence of over-expressed Ral, we crossed the flies with the endogenously EYFP-tag with flies RalCA and also RalWT, using a muscle GAL4 driver to express Ral. The characterization of the distribution of Rabs with muscle RalCA and muscle RalWT is represented in Figures 3.3 and Figure 3.4, respectively.

In figure 3.3 we show the expression of RalCA only in muscle with the expression of the different Rabs EYFP-tag. As described before, the presence of Ral in the constitutively active form in muscle leads to an increased recruitment of exocyst to the NMJ which generates a bigger SSR. Our

analysis did not seem to identify any critical changes in Rabs distribution in the presence of muscle RalCA.

Rab1, Rab3, Rab7, Rab8, Rab10, Rab14, Rab23 and Rab30 seem to have almost the same distribution in NMJ or in muscle when RalCA is present in comparison to what we have seen in without Ral over-expression. Rab1, Rab8 and Rab10 seem to have higher expression at the NMJ, but further quantitative studies have to be performed to confirm this qualitative analysis. On the other hand, Rab4 and Rab5 seem more expressed in the muscle, fact that we didn't observed in the $Rab4^{EYFP}$ and $Rab5^{EYFP}$ situation.

Rab2, Rab6, Rab9, Rab11 and Rab19 seem to have a slightly decrease of expression in muscle and Rab26 and RabX4 seem to have also a slightly decrease but in the NMJ, this could mean that Rab26 and RabX4 might be consumed or metabolized at NMJ. Rab3 and Rab10 seem to have an increase but only in the NMJ, possibly because Rab3 is expressed in synaptic vesicles and synaptic activity activates Ral, making it plausible that Ral can send a retrograde signaling, resulting in higher levels of Rab3 and more synaptic vesicles ready to fuse in active zones. Rab10 is usually associated with vesicle traffic from Golgi to trans-golgi network and GLUT4 vesicle translocation (Bhuin & Roy 2014; Stenmark 2009), in *Drosophila* Rab10 has been associated with branch outgrowth in tracheal terminal cells and in the transport of vesicles to the plasma membrane (Jones et al. 2014). These observations led us to think that it is possible that RalCA increases Rab10 at the synapse, which could contribute to the growth of SSR.

Rab18, Rab27, Rab32, Rab35, Rab39, Rab40, RabX1, RabX2, RabX5 e RabX6 are still not analyzed.

To confirm the results obtained when RalCA is expressed in the muscle, we also analyzed how Rabs are distributed in the presence of muscle RalWT. The results are consistent with what we observed with RalCA, which increases our level of confidence.

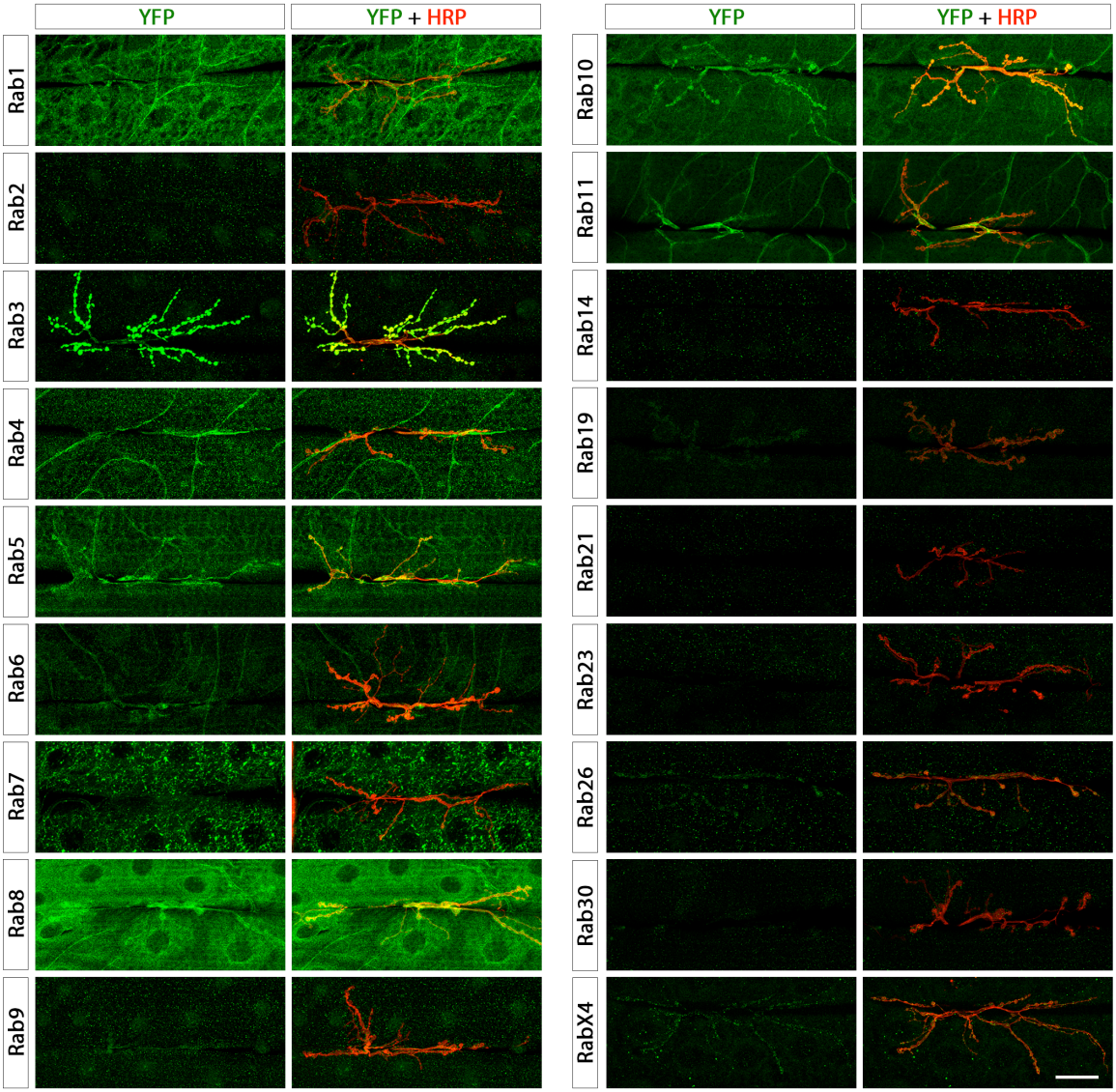


Figure 3.3. Characterization of RabGTPases distribution in *Drosophila* NMJ in the presence of RalCA protein in muscle. The panel shows the distribution of RabGTPases in NMJ of muscles 6/7 of 3rd instar larvae, segment A2 or A3. For all images, scale bar 10 μm. Genotypes are: muscle RalCA;RabX-RNAi.

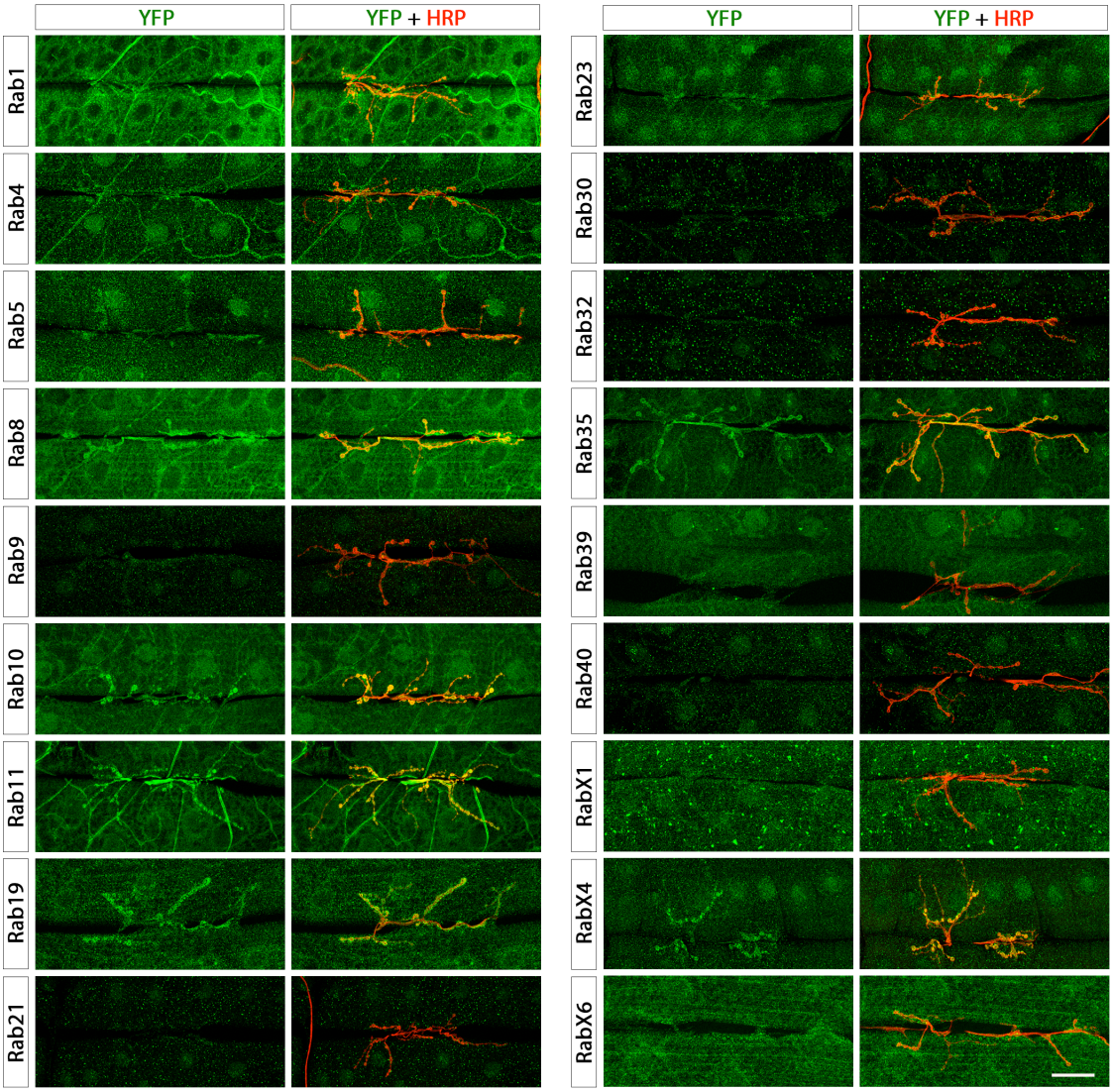


Figure 3.4. Characterization of RabGTPases distribution in *Drosophila* NMJ in the presence of RalWT protein in muscle. The panel show the distribution of RabGTPases in NMJ of muscles 6/7 of 3rd instar larvae, segment A2 or A3. For all images, scale bar 10 μm. Genotypes are: muscle RalWT;RabX-RNAi.

In Figure 3.4 the distribution of Rabs in the presence of muscle RalWT is shown, and is identical to the one represented in Figure 3.3, with muscle RalCA. In the panels is clear to see that in the presence of Ral protein Rab4 and Rab5 are more expressed in the muscle compared to the RabYFP alone, and by the contrary Rab9 is decreased maybe because it might be consumed. Rab19 in the presence of RalCA seems to be decrease but in RalWT and EYFP stock are equal, which might indicate that is a problem with the staining or due to the variability of the animals, or because RalCA has a stronger phenotype. Rab23 in the presence of RalWT has a perinuclear distribution, but we didn't have see that in the other images. Rab35, Rab39, Rab40 and RabX6 in the presence of muscle RalWT seem to have the same distribution as the stock and RabX1 appears to be more punctated than in the stock.

To summarize the results of Rabs expression in NMJ and muscle, we present in Table 3.1 an overview of the expression intensity of all images shown. In the table each Rab genotype is compared in the three conditions from “+” to “+++”, been + expressed and +++ very expressed. And when we didn´t detect any expression pattern, we have a “-“. Red squares in table represent Rabs that still need to be analyzed.

Further experiments have to be done in order to quantify if the intensity of Rabs change in the synapse and muscle in the presence of Ral. Here, we were interested in a qualitative information on Rab GTPase distribution. When we identify candidates to mediate SSR growth, we will do further analysis and quantifications.

Table 3.1. Analysis of Rab-YFP intensity in muscle and NMJ in flies expressing YFP endogenously of the Rab proteins. In the table we show the overview results of the expression of Rabs endogenously and in the presence of RalWT and RalCA in muscle. The results are a comparison between each Rab individually on the tree backgrounds, where the "+"s represent the degree of expression, from + expressed to +++ very expressed, and the "-" for not expressed.

	YFP stock		YFP muscle RalWT		YFP muscle RalCA	
	Muscle	NMJ	Muscle	NMJ	Muscle	NMJ
Rab 1	+++	+	+++	-	++	-
Rab 2	++	-			+	-
Rab 3	-	++			+	+++
Rab 4	-	-	++	-	+	-
Rab 5	-	-	+	-	+	+
Rab 6	+	-			+	-
Rab 7	++	-			++	-
Rab 8	+	++	++	++	+++	++
Rab 9	+	+	+	-	+	-
Rab 10	+	+	++	++	++	++
Rab 11	++	+++	++	+++	+	+
Rab 14	-	-			+	+
Rab 18	+	-				
Rab 19	++	++	++	++	+	+
Rab 21	++	+	++	-	+	-
Rab 23	+	-	++	-	+	-
Rab 26	++	+++			+	+
Rab 27	+	-				
Rab 30	+	-	++	-	+	-
Rab 32	+	-	++	-		
Rab 35	++	+++	++	+++		
Rab 39	++	-	++	-		
Rab 40	+	-	+	-		
Rab X1	+	-	++	-		
Rab X4	+	+++	++	+	+	+
Rab X5						
Rab X6	+	-	++	-		

3.2. Identification of the Rab-GTPases that regulate Ral-dependent SSR growth

Given that Rab GTPases are master regulators of membrane trafficking, we hypothesized that a subset of them could be required to mediate the activity-dependent Ral/Exocyst SSR growth. Teodoro *et al.* (2013) showed that expression of RalWT or RalCA in the muscle leads to Sec5 recruitment to the postsynaptic region, and that results in a larger SSR. To screen for Rabs involved in

this pathway, we used RNAi to reduce the levels of all 28 *Drosophila* Rabs against RalCA and RalWT under a UAS-GAL4 muscle driver. The expectation is that if a given Rab is required for Sec5 recruitment, we would observe a reduction in the levels of Sec5 at the synapse. If the Sec5 staining remains unaltered we can infer that, that Rab didn't play a significant role in SSR growth. Given this, we used RNAi to knock down one Rab at the time and used Sec5 recruitment to the NMJ as readout (Figure 3.5).

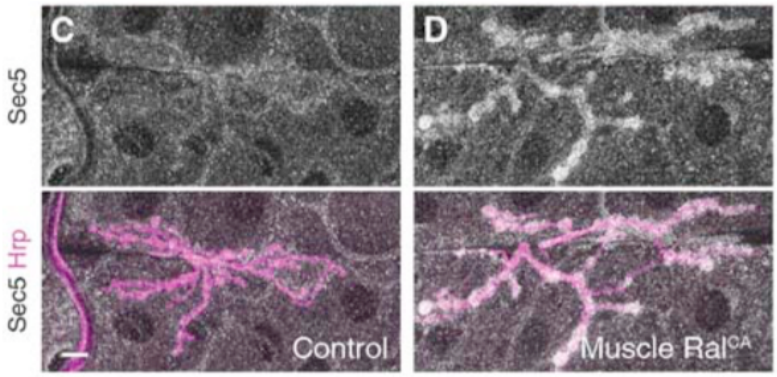


Figure 3.5. Distribution of Sec5 in muscle and NMJ after RalCA expression: Readout used in our RNAi screen. Sec5 is characterized by a ubiquitous distribution is muscle **(C)** and in the presence of more Ral, in this case Ral in the constitutively active form, Sec5 changes is distribution near to NMJ. Adapted from (Teodoro et al. 2013).

3.2.1. Qualitative analyses of the Sec5 recruitment in the presence of RalWT

In the case of RalWT expression in the muscle, we crossed flies that had each of the UAS-Rabs RNAi with UAS-RalWT under a GAL4 muscle driver that simultaneously expresses RalWT and reduces the levels of the Rab to which the RNAi is designed to, specifically in the muscles. The results obtained are described in Figure 3.6.

Our expectation is that we would see alterations in the recruitment of the exocyst to the NMJ if a given Rab is necessary for SSR growth. Our first approach is to qualitatively identify Rab candidates for postsynaptic growth and then performe a quantitative analysis with these candidates. As we can see in the Figure 3.6, and comparing our results to what is described in Teodoro *et al.* (2013) when RalWT is expressed in muscles (Figure 3.5), we can conclude that some Rabs showed less Sec5 recruitment to the NMJ, when compared to the control situation of RalWT only.

From our Rab screen, we chose Rab1, Rab10 and RabX4 as Rabs that appear to show reduced levels of Sec5 recruitment to the NMJ. After Rab1 RNAi expression, we can only see staining for Sec5 in the axon of the NMJ and the branches and boutons had significantly less recruitment of Sec5, which might indicate that Rab1 is necessary for the growth of the SSR. Strikingly, Rab10 didn't show any recruitment of Sec5 to NMJ suggesting that Rab10 might have an important role in postsynaptic growth. RabX4 also showed a decrease in Sec5 recruitment, joining Rab1 and Rab10 in the list of candidates. The other Rabs seem to have normal levels of Sec5 recruitment. This

conclusion is only based on qualitative observations, but to confirm this effect we also tested the Rab RNAi with RalCA under the same GAL4 muscle driver.

In Figure 3.6 Rab2, Rab7, Rab19, Rab21, Rab23, Rab35, Rab39, Rab40, RabX1, RabX2 and RabX5 aren´t present because they aren´t done yet.

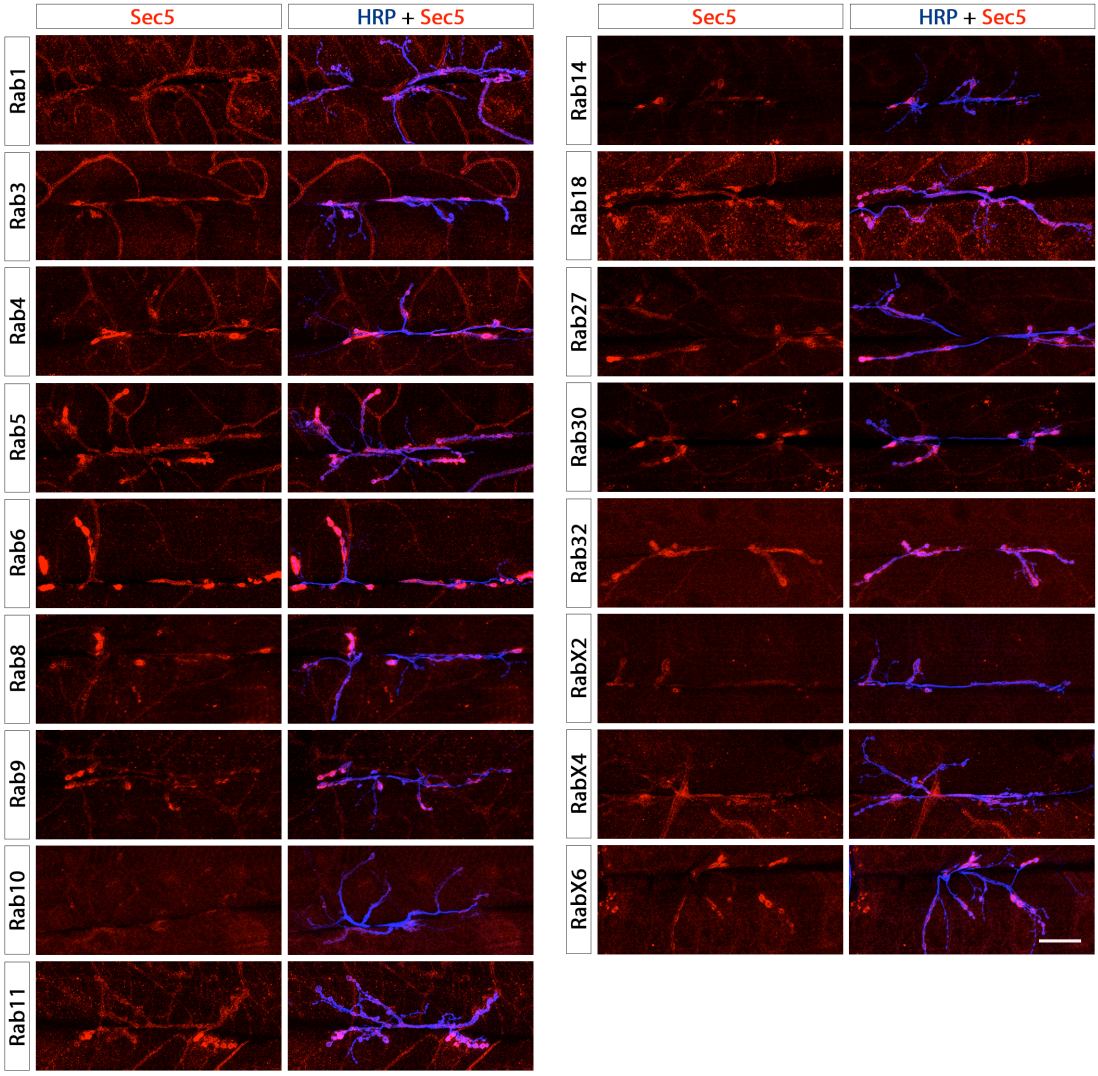


Figure 3.6. Analysis of Sec5 recruitment to the NMJ in the presence of RalWT in the muscle upon knockdown by RNAi. Rab1, Rab10 and RabX4 have affected recruitment of Sec5. For all images, scale bar 10 μm.

3.2.2. Qualitative analyses of Sec5 recruitment in the presence of RalCA

In this case our readout is the same, less Sec5 recruitment will indicate that that Rab might be involved in the growth of the SSR. In the presence of RalCA, Sec5 is more recruited to the NMJ, so any differences should be more identifiable in this background. All the results are consistent in every animal and experiment. The images obtained are described in Figure 3.6. Looking to our results depicted in the panel we can see that Rab1, Rab10 and RabX4 show almost no recruitment to the NMJ, confirming the results that we have obtained with RalWT expression.

In the Figure Rab3, Rab26, Rab30, Rab39, Rab40, RabX1, RabX2 and RabX5 aren´t present because they aren´t done yet.

3.3. Rab1, Rab10 and RabX4 might promote the SSR growth

From the previous screens, we identified Rab1, Rab10 and RabX4 as candidates to regulate Ral/exocyst-dependent SSR growth: this conclusion is based in the qualitative comparison of Sec5 recruitment to the NMJ by immunocytochemistry. In Figure 3.8 we can see the Rabs candidates tested against a control RNAi using a muscle GAL4 driver as a control, and again we can see reduced Sec5 recruitment to the NMJ. This phenotype was consistent in every animal and experiment. We quantified the recruitment of Sec5 to the NMJ as previously described ((Teodoro et al. 2013), and methods). The examples and respective quantification is shown on Figure 3.8. As expected from our qualitative observations, Rab1 and RabX4 RNAi had significantly less Sec5 recruitment to the NMJ, in agreement with a role in SSR growth. However, in the case of Rab10, the quantification showed that Sec5 recruitment to the NMJ was not different from RalCA alone. Despite this, we still considered Rab10 as a good candidate because we observed that there were several cases where we saw entire synaptic branches without any recruitment, immediately adjacent to others that showed normal Sec5 recruitment. It is possible that the RNAi efficacy is not complete, allowing for normal recruitment in some cases and not in others. Also, since the n is still low, it is possible that Rab10 RNAi quantification can achieve significance with a higher number of analyzed NMJs. Alternatively, Rab10 may be required in some parts of the NMJ and not in others, which can be visualized in the images, but would require a different method of quantification, not including the entire NMJ.

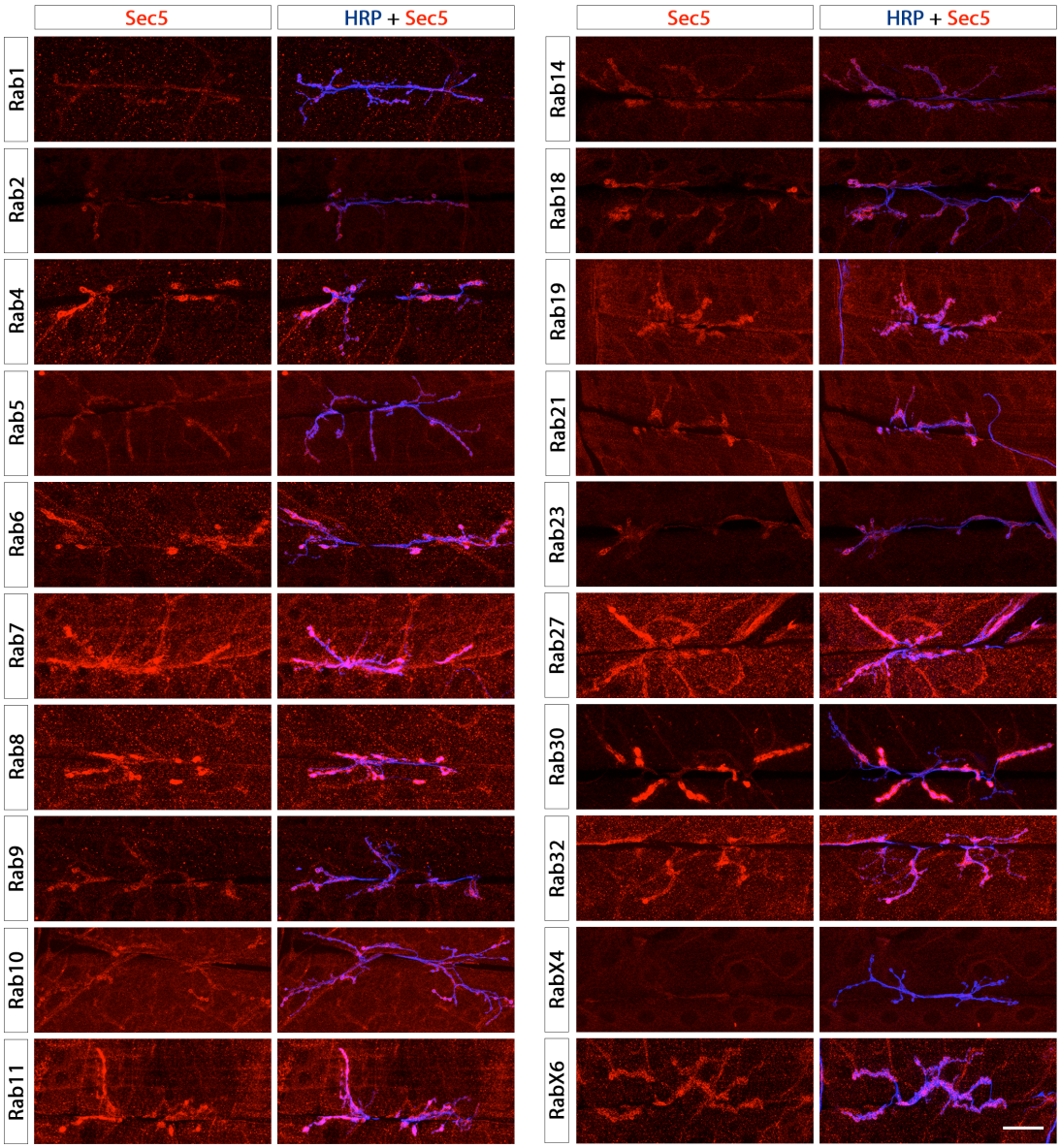
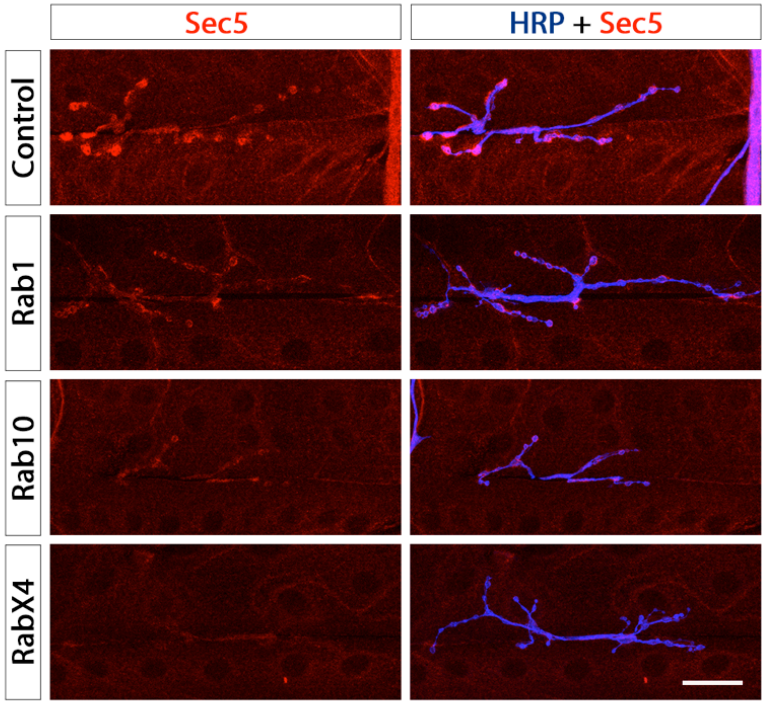


Figure 3.7. Analysis of Sec5 recruitment to the NMJ in the presence of RalCA in the muscle upon knockdown by RNAi. Rab1, Rab10 and RabX4 have affected recruitment of Sec5. For all images, scale bar 10 μm.

A)



B)

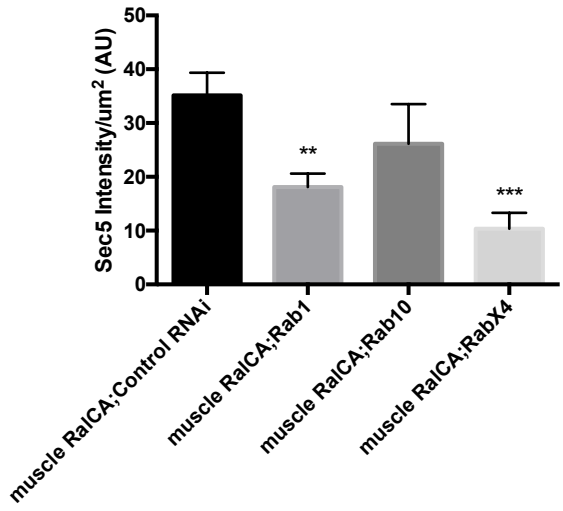


Figure 3.8. Analysis of Sec5 recruitment of Rab candidates in the presence of RalCA in the muscle upon knockdown by RNAi. A) In the panel, control is a GFP RNAi crossed with a RalCA muscle driver. Rab1, Rab10 and RabX4 show defects in the recruitment of Sec5. For all images, scale bar 10 μm. B) Quantification of Sec5 recruitment to the NMJ in each Rab candidate.

The quantification of Sec5 recruitment in the presence of RalCA and Rab RNAi gives support to the idea that these Rabs may be required for the Ral/exocyst pathway thereby contributing to the postsynaptic growth of the SSR.

This three Rabs are implicated in diverse functions and have distinct neuronal roles, described in different organisms. Rab1 is associated to membrane trafficking from ER-to Golgi, and in dopaminergic neurons higher levels of Rab1 can prevent neuronal loss and α-synuclein accumulation

in mammalian neurons (Bhullar et al. 2006). In *Drosophila*, Rab1 marks vesicles from Golgi and previous studies also showed the possible involvement of Rab1 in the extension and retraction of dendritic branches (Ye et al. 2007). Rab10 is associated with the release of neuropeptides from dense core vesicles in *C.elegans* (Sasidharan et al. 2012) and also in axon elongation and dendrite arborization. It is also known that Rab10 and exocyst are conserved, from *C.elegans* to Humans, and in flies Rab10 knock-down also shows less dendritic arborization (Zou et al. 2015). RabX4 in *Drosophila* is responsible for the internalization of transient receptor potential channel (TRPL) into the cell body of the eye (Oberegelsbacher et al. 2011). Interestingly, RabX4 appears to be a Rab that is only present in insects, but it seems that Rab10 and RabX4 in *Drosophila* have diverged from the same branch, which might suggest that these Rabs have similar functions (Pereira-Leal & Seabra 2001).

It is noteworthy to say that it is likely meaningful that Rab1 and Rab10 have been described to play a role in dendritic growth and are candidate in our screen, given that the SSR is the postsynaptic membrane at the NMJ.

3.4. Are Rab candidates required for activity dependent recruitment of Exocyst?

Previous studies demonstrated that the recruitment of Sec5 to the NMJ also occurred when the synapse was stimulated and this recruitment was dependent on Ral. The model proposed was that activity-dependent SSR growth was mediated by the Ral/exocyst pathway (Teodoro et al. 2013). Therefore, if our Rab candidates are involved in this activity-dependent process, we expect to see reduced Sec5 recruitment after synaptic stimulation, in the presence of RNAi against the Rab candidates. To see if activity played a role, we tested the Rab candidates RNAi crossed with UAS-Dicer2 and a GAL4 muscle driver, to quantify the recruitment for Sec5, and subsequently infer about SSR growth.

To mimic a protocol of repeated synaptic depolarization, we used an established protocol that induces the formation of new pre-synaptic immature boutons, and postsynaptic Sec5 recruitment. The presence of these new pre-synaptic boutons, called ghost boutons, serve as a positive control to assure that the stimulation protocol worked. A schematic of this protocol is shown on fig. 3.9. In this experiment larvae were subjected to saline solutions that mimic larval hemolymph in the presence or absence of high K+/high Ca2+ to induce membrane depolarization and to simulate synaptic transmission. This experiment will allow us to infer about the activity-dependence of SSR growth. As described by several groups the stimulation protocol is well established and reproducible (Feng et al. 2004).

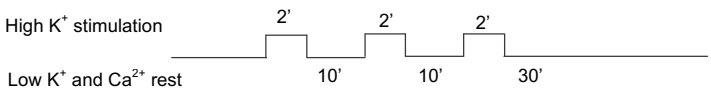


Figure 3.9. Schematic representation of the stimulation paradigm used. 3 stimulations of 3 times 2 minutes, interspaced by 10 minutes. At the end, larvae are fixed and processed for immunocytochemistry.

We tried the stimulation protocol twice in our larvae and we didn´t see any recruitment of Sec5 to the NMJ, like is shown in Figure 3.10. There are several explanations for this: 1) In the first trial there was a pH problem with the solutions that may have been responsible for the failure to see recruitment; 2) because we do all genotypes simultaneously and the recruitment is transient, it is possible that the time between the end of the stimulation and the end of the dissection of the larvae might be too long; 3) despite the two previous explanations, we did not observe the formation of ghost boutons in our control, suggesting that the experiment did not work. In conclusion, I need to optimize the protocol, which is successfully and routinely used in the lab, and repeat the experiment. This will allow us to determine if all 3 Rabs are required for this acute recruitment of Sec5 or only a subset of them. This knowledge will guide us in the dissection of the pathway that contributes to the development of the SSR and for the response to synaptic activity.

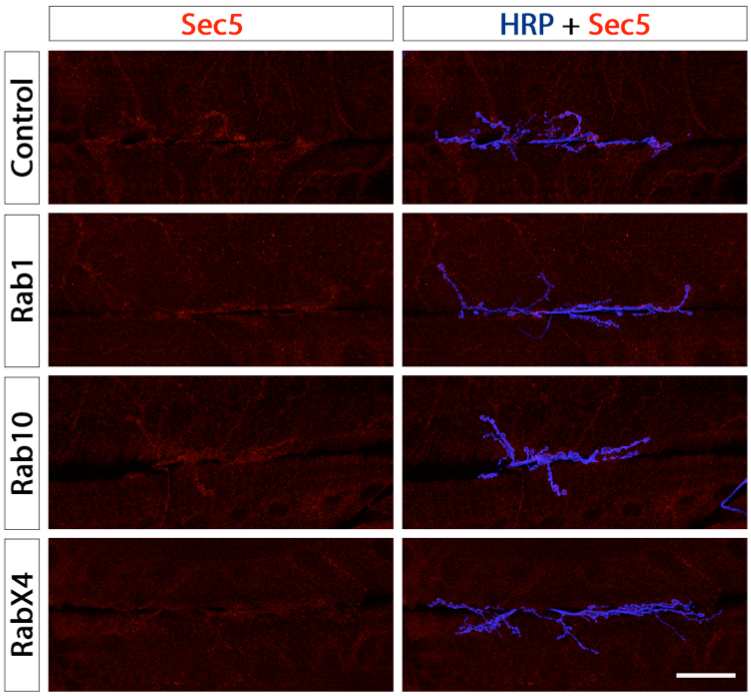


Figure 3.10. Testing Rab candidates for activity-dependent Sec5 recruitment. A) Schematic of stimulation protocol used**. B)** In the panel, control is a GFP RNAi crossed with a Dicer2 Gal4 under muscle driver. The other genotypes are Dicer2; G14-Gal4; UAS-Rab-RNAi For all images, scale bar 10 μm.

3.5. Quantification of the Postsynaptic marker Syndapin

There are two known postsynaptic membrane markers in *Drosophila*, Dlg (Lahey et al. 1994) and Syndapin (Kumar et al. 2009). In *Drosophila*, Syndapin is predominantly localized to the NMJ in 3rd instar larvae and previous studies have established the correlation that more Syndapin at the NMJ represents a bigger SSR. In order to infer about the size of SSR in our candidates, we quantified the levels of Syndapin in control and in muscle RNAi of Rab candidates (Kumar et al. 2009).

In order to quantify the synaptic marker Syndapin, flies with the RNAi of the candidate Rabs were crossed with a fly Dicer2 under a muscle Gal4 driver to see if the amount of Syndapin in the NMJ

was reduced. We acquired images and quantified the intensity of Syndapin at the NMJ. In Figure 3.11. is represented the Syndapin distribution at NMJ in each genotype. In the images we can see that in controls there is more Syndapin than Rab1 and RabX4 RNAi; Rab10 RNAi is lower, but not significantly decreased. Despite this, we think that this lack of significance may derive from a low n; additionally, and posterior to image acquisition, we realized that some of our control images were saturated, masking a stronger phenotype.

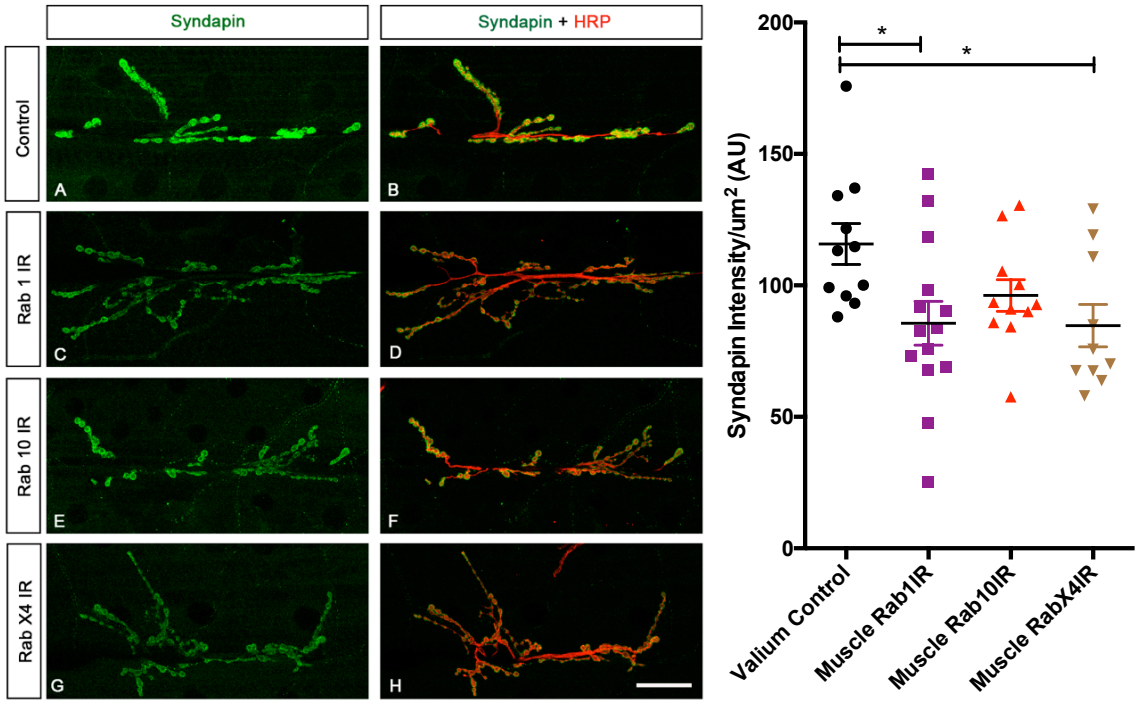


Figure 3.11. Analyses of Syndapin distribution in Rab candidates. (A-F) Images of NMJ 3rd instar larvae, muscle 6/7, segment A2 and A3. (A-D) The intensity of Syndapin staining seems higher in the control (A) and reduced in the other genotypes (B-D). (I) Statistical analyses of Syndapin intensity per area of NMJ. *P<0,01

Since we know that Syndapin is present in the postsynapse of the NMJ, the decreased levels of Syndapin in Rab1 and Rab10 RNAi (and possibly Rab10) intensity might suggest that SSR is smaller than in the control. This result will be confirmed by electron microscopy.

3.6. Visualization of the SSR in Rab candidates RNAi by Transmission Electron Microscopy

The golden standard to measure postsynaptic membrane size is the utilization of Transmission Electron Microscopy (TEM). Therefore, to demonstrate if Rab1, Rab10 and RabX4 mark the vesicles that promote SSR growth, we used TEM to visualize the postsynaptic membrane. We are trying to observe if without Rab1, Rab10 or RabX4 the SSR is thinner than in control.

One of the major challenges in electron microscopy is maintaining the structures the most conserved as possible. In our case we used preparations of whole larva, and we are interested in NMJ

of the muscles 6 and 7, which are amongst the most superficial muscles when a larva is dissected. With this, the access to NMJ and the longitudinal sectioning of the larva seem to be a process relatively simple. But once the body wall of the larva is cut along the middle dorso and it's open, it naturally tends to curl and return to the original shape. Given this, the major difficulty in the optimization of the protocol was having a flat larva embedding so that the sectioning would be as much as possible in the right plan. Without a flat embedding we cannot get intact muscles sections, and cannot visualize entire synaptic boutons and their surrounding SSR.

We tried a number of embedding protocols, and in the times where we succeeded, we were able to acquire muscle sections that had boutons like the ones depicted in Figure 3.12. The quality of the images is still not very good, and more optimizations or post-staining have to be performed in order to be able to acquire many images of all the genotypes of interest, so we can quantify SSR thickness. At this point the question "Is the SSR thinner when we knockdown the candidate Rabs?", is still an open question.

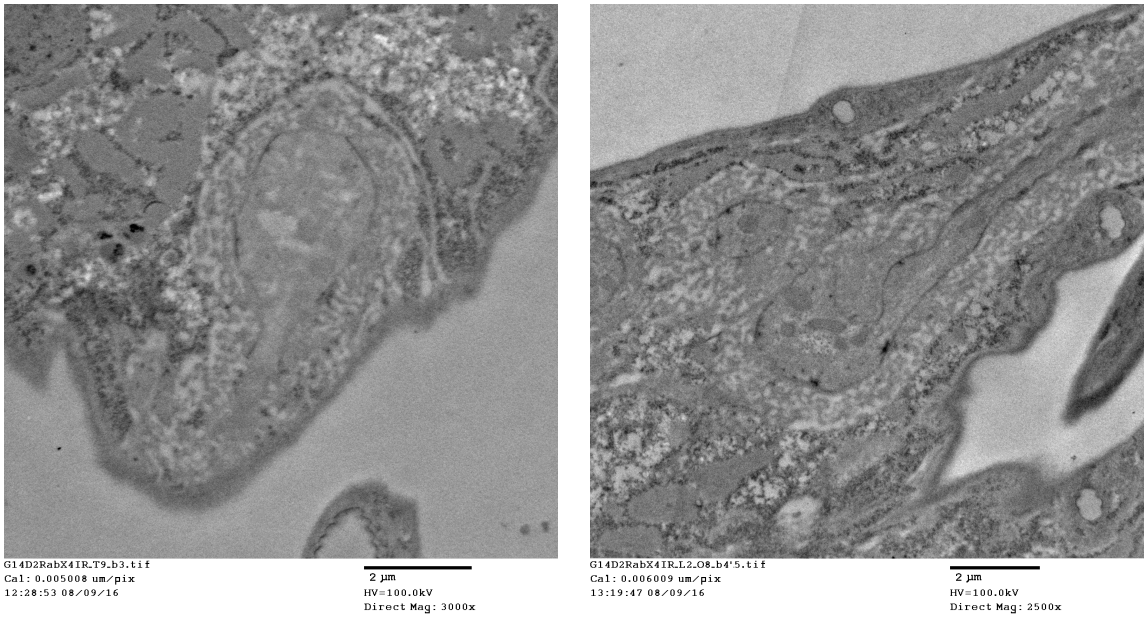


Figure 3.12. TEM images of Rab candidate RNAi, RabX4. In both images we can see a synaptic bouton surrounded by the postsynaptic membrane, the SSR. Comparing with what is described by Teodoro et al. 2013 and in introduction, these synaptic boutons seem to have a thinner SSR than wild type, but we can only speculate because quantifications have to be performed.

Chapter 4. Conclusions and Future Perspectives

Neurons are one of the cell types that show more plasticity in the presence of changing levels of synaptic activity. For the formation and maintenance of neuronal structures, membrane trafficking is necessary. One of the best-characterized regulators of membrane traffic are small GTPases, like Ral and Rab GTPases. Given this, understanding the mechanisms by which small GTPases convert synaptic activity in structural plasticity and growth can give us insights into several neuronal processes and what happens when these pathways are perturbed and disease occurs.

At the *Drosophila* neuronal muscular junctions, the postsynapse is a membranar structure that shows plasticity in response to a stimulus, and it is described that by the activation of the complex Ral/Exocyst vesicles are recruited and this might contribute to SSR growth. Given this, we focused this work in understanding which Rab GTPases contribute to postsynaptic growth and plasticity, by looking for the endogenous distribution of them and knocking down each one at the time to look for alterations in the recruitment of the complex Ral/exocyst to the NMJ. Three candidates were identified, Rab1, Rab10 and RabX4. This three Rabs have been associated with different processes like Golgi secretion in the case of Rab1; Rab10 is associated with recycling pathways and RabX4 is an insect Rab that not much is known.

Combining these results with what is described in literature we can hypothesize that SSR growth is dependent on different pools of vesicles that are transported from different organelles to the synapse. Given this, and considering the model proposed by Teodoro et al 2013, we proposed the model described in Figure 4.1.

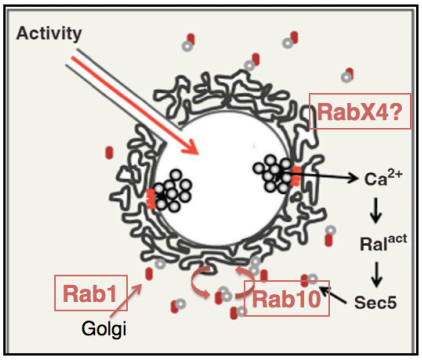


Figure 4.1. Model for SSR growth in a Ral/Exocyst dependent manner. We hypothesize that different pools of vesicles contribute to plasticity growth of SSR. Rab1 might be promoting the traffic of vesicles from Golgi, Rab10 in involved in recycling pathways and RabX4 is an insect specific Rab with unknown function.

These three pools of vesicles might indicate different ways of Rab response to activity. It is possible that Rab1 vesicles will be more important for continuous SSR growth, while Rab10 would be used for acute situations as in the case of an acute stimulus. Our experiments will distinguish these possibilities.

Another pending question is “what is the biological function of SSR”? To answer it, we have been developing a system that allow us to film adult flies and larvae to calculate, among others parameters, velocity and to characterize locomotion parameters. The setup is under development and

we hope to identify parameters that will vary with genotypes, allowing us to combine this information with other analysis and predict how the SSR size contributes to behavior.

Chapter 5. References

Ashburner, M. & Roote, J., 2007. Maintenance of a Drosophila laboratory: general procedures. *CSH protocols*, 2007, p.pdb.ip35.

Bahadoran, P. et al., 2001. Rab27a: A key to melanosome transport in human melanocytes. *Journal of Cell Biology*, 152(4), pp.843–849.

Bhuin, T. & Roy, J.K., 2014. Rab proteins: The key regulators of intracellular vesicle transport. *Experimental Cell Research*, 328(1), pp.1–19.

Bhullar, B. et al., 2006. a-Synuclein Blocks ER-Golgi Traffic and Rab1 Rescues Neuron Loss in Parkinson's Models. *Science*, 452(March), pp.324–328.

Bozzola, J.J. & Russell, L.D., 1999. *Electron Microscopy: Principles and Techniques for Biologists*,

Brand, A.H. & Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development (Cambridge, England)*, 118(2), pp.401–15.

Brent, J., Werner, K. & McCabe, B.D., 2009. Drosophila larval NMJ immunohistochemistry. *Journal of visualized experiments : JoVE*, pp.4–5.

Caygill, E.E. & Brand, A.H., 2016. The GAL4 System: A Versatile System for the Manipulation and Analysis of Gene Expression. In pp. 33–52. Available at: http://link.springer.com/10.1007/978-1-4939-6371-3.

Celniker, S.E., 2000. The Drosophila genome. *Current Opinion in Genetics & Development*, 10(6), pp.612–616.

Chan, C.C. et al., 2011. Systematic discovery of Rab GTPases with synaptic functions in Drosophila. *Current Biology*, 21(20), pp.1704–1715.

Chen, X.-W. et al., 2011. Exocyst function is regulated by effector phosphorylation. *Nature cell biology*, 13(5), pp.580–8.

Colgan, L.A. & Yasuda, R., 2014. Plasticity of Dendritic Spines: Subcompartmentalization of Signaling. *Annual Review of Physiology*, 76(1), pp.365–385.

Colicelli, J., 2010. Human RAS Superfamily Proteins and Related GTPases. *Molecular Biology*, 2004(250), pp.1–53.

Collins, C.A. & DiAntonio, A., 2007. Synaptic development: insights from Drosophila. *Current Opinion in Neurobiology*, 17(1), pp.35–42.

D'Adamo, P. et al., 2014. RAB GTPases and RAB-interacting proteins and their role in the control of cognitive functions. *Neuroscience and Biobehavioral Reviews*, 46(P2), pp.302–314.

van Dam, E.M. & Robinson, P.J., 2006. Ral: Mediator of membrane trafficking. *International Journal of Biochemistry and Cell Biology*, 38(11), pp.1841–1847.

Demerec, M. & Kaufman, B.P., 1996. Drosophila guide. , p.46.

Duffy, J.B., 2002. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis (New York, N.Y. : 2000)*, 34(1–2), pp.1–15.

Dunst, S. et al., 2015. Endogenously Tagged Rab Proteins: A Resource to Study Membrane Trafficking in Drosophila. *Developmental Cell*, 33(3), pp.351–365.

Echard, A. et al., 1998. Interaction of a Golgi-associated kinesin-like protein with Rab6. *Science (New York, N.Y.)*, 279(5350), pp.580–585. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9438855.

Elliott, D.A. & Brand, A.H., 2008. The GAL4 system : a versatile system for the expression of genes. *Methods in molecular biology (Clifton, N.J.)*, 420, pp.79–95.

Feig, L.A., 2003. Ral-GTPases: Approaching their 15 minutes of fame. *Trends in Cell Biology*, 13(8), pp.419–425.

Feng, Y., Ueda, A. & Wu, C.-F., 2004. A MODIFIED MINIMAL HEMOLYMPH-LIKE SOLUTION, HL3.1, FOR PHYSIOLOGICAL RECORDINGS AT THE NEUROMUSCULAR JUNCTIONS OF NORMAL AND MUTANT DROSOPHILA LARVAE. *Journal of Neurogenetics*, 18(2), pp.377–402.

Finger, F.P., Hughes, T.E. & Novick, P., 1998. Sec3p is a spatial landmark for polarized secretion in budding yeast. *Cell*, 92(4), pp.559–571.

Fire, A. et al., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391(6669), pp.806–811.

Friedrich, G. a, Hildebrand, J.D. & Soriano, P., 1997. The secretory protein Sec8 is required for paraxial mesoderm formation in the mouse. *Developmental biology*, 192(2), pp.364–74.

Gerges, N.Z. et al., 2006. Dual role of the exocyst in AMPA receptor targeting and insertion into the postsynaptic membrane. *The EMBO Journal*, 25(8), pp.1623–1634.

Graf, E.R. et al., 2009. Rab3 Dynamically Controls Protein Composition at Active Zones. *Neuron*, 64(5), pp.663–677. Available at: http://dx.doi.org/10.1016/j.neuron.2009.11.002.

Gramates, L. & Budnik, V., 1999. Assembly and Maturation of the Drosophila Larval Neuromuscular Junction. *International Review of Neurobiology*, 43, pp.93–117.

Grindstaff, K.K. et al., 1998. Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell*, 93(5), pp.731–740.

Guo, W. et al., 1999. The exocyst is an effector for Sec4P, targeting secretory vesicles to sites of exocytosis. *EMBO Journal*, 18(4), pp.1071–1080.

Guo, W., Tamanoi, F. & Novick, P., 2001. Spatial regulation of the exocyst complex by Rho1 GTPase. *Nature cell biology*, 3(4), pp.353–360.

Harris, K.P. & Littleton, J.T., 2015. Transmission, development, and plasticity of synapses. *Genetics*, 201(2), pp.345–375.

Hazuka, C.D. et al., 1999. The sec6/8 complex is located at neurite outgrowth and axonal synapse-assembly domains. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(4), pp.1324–1334.

He, B. et al., 2007. Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. *The EMBO journal*, 26(18), pp.4053–4065.

Heider, M.R. & Munson, M., 2012. Exorcising the Exocyst Complex. *Traffic*, 13(7), pp.898–907.

Hill, C. et al., 2015. Plasticity and Second Messengers During Synapse Development. *Proceedings of SPIE--the International Society for Optical Engineering*, 73(4), pp.389–400.

Hofer, F., Berdeaux, R. & Martin, G.S., 1998. Ras-independent activation of Ral by a Ca(2+)-dependent pathway. *Current biology : CB*, 8(14), pp.839–42.

Hsu, S.C. et al., 1998. Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. *Neuron*, 20(6), pp.1111–1122.

Hume, A.N. et al., 2007. Rab27a and MyoVa are the primary Mlph interactors regulating melanosome transport in melanocytes. *Journal of cell science*, 120, pp.3111–3122.

Jenkins, D. et al., 2007. RAB23 Mutations in Carpenter Syndrome Imply an Unexpected Role for Hedgehog Signaling in Cranial-Suture Development and Obesity. *The American Journal of Human Genetics*, 80(6), pp.1162–1170.

Jones, T.A. et al., 2014. Exocyst-mediated membrane trafficking is required for branch outgrowth in Drosophila tracheal terminal cells. *Developmental Biology*, 390(1), pp.41–50. Available at: http://dx.doi.org/10.1016/j.ydbio.2014.02.021.

Junction, N. et al., 2016. Rab3-GEF Controls Active Zone Development at. , 3(April), pp.1–18.

Kennedy, M.J. et al., 2010. Syntaxin-4 defines a domain for activity-dependent exocytosis in dendritic spines. *Cell*, 141(3), pp.524–535.

Kessels, M.M. & Qualmann, B., 2002. Syndapins integrate N-WASP in receptor-mediated endocytosis. *EMBO Journal*, 21(22), pp.6083–6094.

Ketel, K. et al., 2016. A phosphoinositide conversion mechanism for exit from endosomes. *Nature*, 529(7586), pp.408–412.

Kumar, V. et al., 2009. Syndapin Promotes Formation of a Postsynaptic Membrane System in

Drosophila. *Molecular Biology of the Cell*, 20, pp.2254–2264.

Lahey, T. et al., 1994. The drosophila tumor suppressor gene dlg is required for normal synaptic bouton structure. *Neuron*, 13(4), pp.823–835.

Lazar, T., Götte, M. & Gallwitz, D., 1997. Vesicular transport: how many Ypt/Rab-GTPases make a eukaryotic cell? *Trends in biochemical sciences*, 22(12), pp.468–472.

Liu, J. & Guo, W., 2012. The exocyst complex in exocytosis and cell migration. *Protoplasma*, 249(3), pp.587–597.

Mark, B.L., Jilkina, O. & Bhullar, R.P., 1996. Association of Ral GTP-binding protein with human platelet dense granules. *Biochemical and biophysical research communications*, 225(1), pp.40–6.

Martin-Urdiroz, M. et al., 2016. The Exocyst Complex in Health and Disease. *Frontiers in Cell and Developmental Biology*, 4(April), pp.1–22.

Matern, H.T. et al., 2001. The Sec6/8 complex in mammalian cells: characterization of mammalian Sec3, subunit interactions, and expression of subunits in polarized cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98(17), pp.9648–53.

Menon, K.P., Carrillo, R.A. & Zinn, K., 2013. Development and plasticity of the Drosophila larval neuromuscular junction. *Wiley interdisciplinary reviews. Developmental biology*, 2(5), pp.647–70.

Mizuno-Yamasaki, E., Rivera-Molina, F. & Novick, P., 2012. GTPase networks in membrane traffic. *Annual review of biochemistry*, 81, pp.637–59.

Mizuno, S. et al., 2015. Peri-implantation lethality in mice carrying megabase-scale deletion on 5qc3.3 is caused by Exoc1 null mutation. *Scientific Reports*, 5(August), p.13632.

Mohammadi, S. & Isberg, R.R., 2013. Cdc42 interacts with the exocyst complex to promote phagocytosis. *Journal of Cell Biology*, 200(1), pp.81–93.

Moskalenko, S. et al., 2003. Ral GTPases Regulate Exocyst Assembly through Dual Subunit Interactions. *Journal of Biological Chemistry*, 278(51), pp.51743–51748.

Moskalenko, S. et al., 2002. The exocyst is a Ral effector complex. *Nature cell biology*, 4(1), pp.66–72.

Munson, M. & Novick, P., 2006. The exocyst defrocked, a framework of rods revealed. *Nature Structural & Molecular Biology*, 13(7), pp.577–581.

Murthy, M. et al., 2003. Mutations in the Exocyst Component Sec5 Disrupt Neuronal Membrane Traffic , but Neurotransmitter Release Persists. , 37, pp.433–447.

Novick, P., 2016. Regulation of Membrane Traffic by Rab GEF and GAP Cascades. *Small GTPases*,

1248(July), pp.00–00.

Novick, P., Field, C. & Schekman, R., 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell*, 21(1), pp.205–215.

Novick, P. & Guo, W., 2002. Ras family therapy: Rab, Rho and Ral talk to the exocyst. *Trends in Cell Biology*, 12(6), pp.247–249.

Oberegelsbacher, C. et al., 2011. The Drosophila TRPL ion channel shares a Rab-dependent translocation pathway with rhodopsin. *European Journal of Cell Biology*, 90(8), pp.620–630.

Oh, E. & Robinson, I., 2012. Barfly: Sculpting membranes at the Drosophila neuromuscular junction. *Developmental Neurobiology*, 72(1), pp.33–56.

Park, M. et al., 2006. Plasticity-Induced Growth of Dendritic Spines by Exocytic Trafficking from Recycling Endosomes. *Neuron*, 52(5), pp.817–830.

Peng, J. et al., 2004. Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. *The Journal of biological chemistry*, 279(20), pp.21003–21011.

Pereira-Leal, J.B. & Seabra, M.C., 2001. Evolution of the Rab family of small GTP-binding proteins. *Journal of molecular biology*, 313(4), pp.889–901.

Pérez-Otaño, I. et al., 2006. Endocytosis and synaptic removal of NR3A-containing NMDA receptors by PACSIN1/syndapin1. *Nature neuroscience*, 9(5), pp.611–21.

Pfeffer, S. & Aivazian, D., 2004. TARGETING RAB GTPases TO DISTINCT MEMBRANE COMPARTMENTS. , 5(November), pp.886–896.

Pfeffer, S.R., 2013. Rab GTPase regulation of membrane identity. *Current Opinion in Cell Biology*, 25(4), pp.414–419.

Pfeffer, S.R., 1994. Rab GTPases: Master regulators of membrane trafficking. *Current Opinion in Cell Biology*, 6(4), pp.522–526.

Pfenninger, K.H., 2009. Plasma membrane expansion: a neuron's Herculean task. *Nature reviews. Neuroscience*, 10(4), pp.251–61.

Polzin, A. et al., 2002. Ral-GTPase influences the regulation of the readily releasable pool of synaptic vesicles. *Molecular and cellular biology*, 22(6), pp.1714–1722.

Reiter, L.T. et al., 2001. A Systematic Analysis of Human Disease-Associated Gene Sequences In. *Genome Research*, pp.1114–1125.

Ruiz-Cañada, C. & Budnik, V., 2006. Introduction on The Use of The Drosophila Embryonic/Larval Neuromuscular Junction as A Model System to Study Synapse Development and Function, and A Brief Summary of Pathfinding and Target Recognition. *International Review of Neurobiology*,

75(6), pp.1–31.

Sasidharan, N. et al., 2012. RAB-5 and RAB-10 cooperate to regulate neuropeptide release in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America*, 109(46), pp.18944–9.

Schoch, S. et al., 2002. RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature*, 415(6869), pp.321–6.

Shen, D. et al., 2013. The synaptobrevin homologue Snc2p recruits the exocyst to secretory vesicles by binding to Sec6p. *Journal of Cell Biology*, 202(3), pp.509–526.

Shen, K. & Cowan, C.W., 2010. Guidance molecules in synapse formation and plasticity. *Cold Spring Harbor perspectives in biology*, 2(4).

Shirakawa, R. & Horiuchi, H., 2015. Ral GTPases: Crucial mediators of exocytosis and tumourigenesis. *Journal of Biochemistry*, 157(5), pp.285–299.

Sidhu, R.S., Clough, R.R. & Bhullar, R.P., 2005. Regulation of phospholipase C-delta1 through direct interactions with the small GTPase Ral and calmodulin. *The Journal of biological chemistry*, 280(23), pp.21933–21941.

Stenmark, H., 2009. Rab GTPases as coordinators of vesicle traffic. *Nature reviews. Molecular cell biology*, 10(8), pp.513–25.

Sugihara, K. et al., 2002. The exocyst complex binds the small GTPase RalA to mediate filopodia formation. *Nature Cell Biology*, 4(1), pp.73–78.

Takai, Y. et al., 2001. Small GTP-binding proteins. *Physiological reviews*, 81(1), pp.153–208.

Tanaka, T., Goto, K. & Iino, M., 2016. Diverse Functions and Signal Transduction of the Exocyst Complex in Tumor Cells. *Journal of Cellular Physiology*, (August 2016).

Teodoro, R.O. et al., 2013. Ral mediates activity-dependent growth of postsynaptic membranes via recruitment of the exocyst. , (November 2012), pp.2039–2055.

TerBush, D.R. et al., 1996. The Exocyst is a multiprotein complex required for exocytosis in Saccharomyces cerevisiae. *The EMBO journal*, 15(23), pp.6483–6494.

Tojima, T. & Kamiguchi, H., 2015. Exocytic and endocytic membrane trafficking in axon development. *Development Growth and Differentiation*, 57(4), pp.291–304.

Touchot, N., Chardin, P. & Tavitian, A., 1987. Four additional members of the ras gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. *Proceedings of the National Academy of Sciences of the United States of America*, 84(23), pp.8210–4.

Utani, A., 2010. Laminin ??3 chain-derived peptide promotes keratinocyte migration and wound closure: Clustering of syndecan-4 and integrin ??1. *Seikagaku*, 82(4), pp.327–331.

Verhoeven, K. et al., 2003. Mutations in the small GTP-ase late endosomal protein RAB7 cause Charcot-Marie-Tooth type 2B neuropathy. *Am J Hum Genet*, 72(3), pp.722–727.

Wang, K.L. & Roufogalis, B.D., 1999. Ca2-/Calmodulin Stimulates GTP Binding to the Ras-related Protein Ral-A. , pp.14525–14528.

Wang, L., Li, G. & Sugita, S., 2004. RalA-Exocyst Interaction Mediates GTP-dependent Exocytosis. *Journal of Biological Chemistry*, 279(19), pp.19875–19881.

Weigmann, K. et al., 2003. FlyMove – a new way to look at development of Drosophila. *Trends in Genetics*, 19(6), pp.306–310.

Wickner, W. & Schekman, R., 2008. Membrane fusion. *Nature structural & molecular biology*, 15(7), pp.658–64.

Wu, B. & Guo, W., 2015. The Exocyst at a Glance. *Journal of Cell Science*, 128(16), pp.2957–2964.

Wu, S. et al., 2005. Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. *Nature structural & molecular biology*, 12(10), pp.879–85.

Yamamoto-Hino, M. & Goto, S., 2013. In Vivo RNAi-based screens: Studies in model organisms. *Genes*, 4(4), pp.646–665.

Ye, B. et al., 2007. Growing Dendrites and Axons Differ in Their Reliance on the Secretory Pathway. *Cell*, 130(4), pp.717–729.

Yu, I.-M. & Hughson, F.M., 2010. Tethering factors as organizers of intracellular vesicular traffic. *Annual review of cell and developmental biology*, 26, pp.137–156.

Zerial, M. & McBride, H., 2001. Rab proteins as membrane organizers. *Nature reviews. Molecular cell biology*, 2(2), pp.107–117.

Zhang, J. et al., 2007. Thirty-one flavors of Drosophila Rab proteins. *Genetics*, 176(2), pp.1307–1322.

Zou, W. et al., 2015. RAB-10-Dependent Membrane Transport Is Required for Dendrite Arborization. *PLoS Genetics*, 11(9), pp.1–28.